

A suitable case for education

WE ARE back in the what's-wrong-with-British-industry business again. After a lull of every bit of twelve months during which there has not been a single weighty document for us to consider in all its worthy detail, the Department of Industry has, with some connivance from the Department of Education and Science, put out a discussion paper, *Industry, education and management* (free from the Department of Industry), which is aimed to "prompt discussion and, more important, action" on the quality of industrial management. The paper fully acknowledges that the poor productivity showing of British industry is by no means exclusively the result of bad management, but tries at least to isolate just a limited number of aspects of the problem—those concerned with technological management by graduates in Britain's manufacturing industries.

If the paper is familiar stuff in many ways, being (so it seems) a distillation of generally expressed views and well-known pieces of information rather than an expression of radically new thoughts, it still serves a useful purpose of confronting the nation yet again with the woeful way in which it doesn't care about engineers and engineering, somehow regarding the creation of wealth as being a relatively inferior occupation, to be avoided if anything else (preferably involving only paperwork) can be found. The statistics tell the story starkly. As recently as 1969, 40% of graduates entering home employment (excluding the medical sciences) went into manufacturing industries. Now the figure hovers around 25%. At the same time growth in the number of first-degree graduates in science and engineering all but stopped after 1968. Furthermore, tables of after-tax salaries adjusted, as well as can be, for the cost of living in different countries show that top executives on the manufacturing and engineering side are consistently worse paid in the UK than in any other European country. Managerial salaries in industry have lagged very seriously behind inflation and as a result of that and very heavy taxation the salary differential between top executives and average weekly-

wage earners is much lower in the UK than anywhere else in Europe save super-egalitarian Sweden.

Nor is there much but gloom in those perceptions of industry which cannot be turned into figures. Television nightly presents the British industrial scene as a battleground between aloof management and irresponsible unions over issues of the most trifling character. Companies are often thought to be run by boards remote from the factory both geographically and in comprehension. Industrial jobs are reckoned to be much more vulnerable than those in the professions, government or academe. Who then is for manufacturing industry; most of all who wants to be on the production side—the Cinderella of the engineering profession?

All of this has, in one form or another, been talked about *ad nauseam* in the past few years without as yet any perceptible improvements, and more doses of it are on the way with a British Association report and then the government's own enquiry into the status of engineers. But in the end it is the educational system which must take up the running, if only because it has a flexibility within it that industry does not have. The first step must be to persuade more intelligent young people at least to sample engineering at school and in university. There seems to be some evidence that young people go into engineering on the basis of the impression that local industry makes on them, either through family connections or simply through exposure to nearby activity. It ought then to be the duty of schools to make sure that local industry really makes itself accessible and explains what it is about to school-children.

Schools cannot expect to do this without one or two of their staff having a more than passing acquaintance with industry themselves. And universities should make sure that engineering is better taught, with richer experience. How many students' summers are wasted by selling ice-cream when they should be learning what industry is about by being in it—and how many universities go to much trouble to persuade industry to find summer jobs for their students? □



The world balance: judge with care

Robert Press delivers his verdict on the 1977 Yearbook of the Stockholm International Peace Research Institute.

THE 1977 Yearbook has not avoided the dangers about which SIPRI was warned in *Nature* editorials in 1975 (17 April) and 1976 (8 July). It must be regretted by any objective student in the armament and disarmament community that such an otherwise impressive compilation of military statistics should at times depart from the detachment and neutrality that SIPRI could so usefully provide. Various points are made and opinions or beliefs are expressed which, in the absence of being argued through, have too often an appearance of slanting objective information in a manner which one had hoped an organisation with SIPRI's purpose would avoid.

In analysing the non-nuclear issues, the 1977 Yearbook appears to be very much more concerned with the armaments side of the coin than with the disarmament side. The dimensions of a problem are often stated and supported by a wealth of statistical information but with little or nothing about what is being done to tackle the problem. The Yearbook gives only cursory coverage to the work of the Conference of the Committee on Disarmament (CCD); some analysis of the 1976 sessions would have been useful to the reader rather than merely five brief references to CCD plus lists of working papers tabled in 1976. A SIPRI assessment of the UN Special Session on Disarmament, due to take place in New York in May–June 1978 would also have been welcome. Although this is of great importance as a non-aligned initiative, it is disposed of in a three line reference in an appendix and it is likely to be over by the time the 1978 Yearbook appears.

The Yearbook performs a most useful service in publishing arms trade registers. The section on world military expenditure contains a mass of detailed and interesting data, though lacking in steps to reduce it. One looks in vain, in the index, for a reference to the UN Reduction of Military Budgets (ROMB) exercise, or for the Economic and Social Consequences of the Arms Race (ESCAR). Neither is there reference to a significant US piece of legislation in the International Security Assistance and Arms Export Control Act of June 1976, nor to the UN Secretary-General's study in an 1976 experts' report on the measurement and international reporting of military expenditures. Also lacking is a reference to the Japanese initiative (in the 1976 United Nations General Assembly) with a draft resolution calling for a UN study of international arms transfers. It is significant that this resolution was supported by the West but frustrated by the non-aligned countries, many of whom resent what they considered to be attempts to restrict their capacity to defend themselves.

The most significant point which emerges from a study of the tables on pages 222–3 is the increase in the military expenditure of Third World countries, whose share of global military expenditure appears to have increased from 6% in 1966 to 15% in 1976. One would have welcomed some explanation of this rapid growth. It is not all due to increased arms transfers since these still represent only 14.5% of the Third World's military expenditure. Granted that the arms trade with Third World countries is the area where the greatest increase has occurred, inclusion of trade between industrialised nations would not only have been useful to complete the global picture but would have provided a useful basis for comparison between SIPRI's figures and the United States Arms Control and Disarmament Agency (ACDA) annual volume on world military expenditures and arms transfers.

In discussing developments in nuclear arsenals it is asserted that "the most dangerous development in strategic weapons is the continuous improvement of the accuracy of warhead delivery". One would have welcomed an argued defence for this assertion against the case for greatest accuracy possible for all targets other than cities. In this connection it is arguable (but again not argued) whether, as asserted, military R&D is the "one activity which makes possible the arms race" or whether such R&D is an inevitable consequence of a prior political situation. The latter possibility appears to be recognised in the chapter on international nuclear transactions where it is said that a shift of emphasis "towards the root causes of nuclear proliferation is urgently needed" and that "the best way to slow down nuclear proliferation would be for the nuclear weapon states to show by their actions that they are willing to downgrade the political and military role of nuclear weapons".

The chapter dealing with the 'main events and concerns of the year' (1976) inevitably refers to "the new surge of concern about the possible proliferation of nuclear weapons to countries which do not already have these weapons" and it looks upon the year as a "a particularly meagre year for efforts to slow down the Soviet–US arms race to limit armaments". Even the Peaceful Nuclear Explosions Treaty, signed in 1976, would in SIPRI's view, like the 1974 Threshold Test Ban Treaty, be better to remain unratified since it may only delay a comprehensive test ban. One can readily agree that any treaty which allows PNE to continue cannot be truly described as a comprehensive test ban treaty.

Given the importance of the whole issue of nuclear weapon proliferation, the confusion and emotion that arises in discussing the inter-relationship of civil nuclear capability and the potential diversion to military application, the Yearbook's treatment of the proliferation issue is at times less precise and objective than one could hope for from SIPRI. For example, in a reference to the proliferation of uranium enrichment technology to non-nuclear weapon countries,

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"in particular to FR Germany, the Netherlands and South Africa", the Yearbook fails to point out that these three countries were themselves simultaneously developing isotope separation technology. This may be "proliferation" in one sense but not in the generally accepted sense of commercial transfer of know-how and equipment from nuclear haves to have-nots.

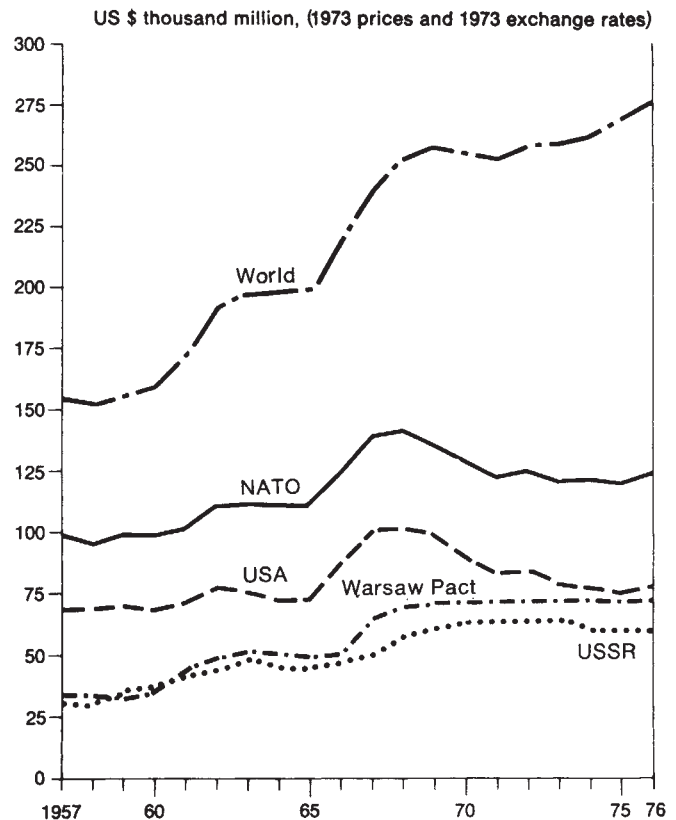
It is claimed that while the Soviet Union followed the USA, UK and Canada in negotiating bilateral agreements, they did so in "a more cautious manner", following their dispute with China, and they have been "rather restricted in their nuclear transactions . . . whereas some Western European countries have adopted more aggressive commercial practices". Yet in the same chapter it is noted that when Canada permanently cut off its nuclear sales to India, two years after the Indian nuclear explosion (1974), "the Soviet Union agreed to sell 200 tons of heavy water to India instead". There is no mention that this action was taken before a safeguards agreement with IAEA had been negotiated. The Yearbook has no hesitation in pointing to a USA offer "to sell nuclear reactors to Israel and Egypt even though these States are not subject to NPT Safeguards" without adding, for the readers' information, anything about the offer being subject to IAEA or US safeguards. Reference to the major nuclear suppliers' efforts to minimise the risk of diversion of nuclear equipment and material is accompanied by the comment "which they are so eager to supply", without any acknowledgement of those countries' obligations under the NPT.

Incidentally, under a sub-heading of nuclear explosives as by-products of a peaceful nuclear power programme, in Fig. 1.1, one wonders about the value of the statement: "The marginal cost of so producing a nuclear explosive device may be no more than a few hundred thousand dollars". The reader may well wonder whether this is an informed estimate and what is meant by "marginal cost"; if not an informed estimate, does the statement render any positive service to the cause of non-proliferation? However, in this context, a more realistic note is struck when it is observed that "a lack of access to a commercial reprocessing plant would not necessarily prevent the proliferation of nuclear weapons to countries which take the decision to acquire them directly".

On 'the plutonium problem', the Yearbook falls into the usual trap of failing to separate the 'nth country' problem from the potential 'terrorist' problem and therefore fails to distinguish between the different kinds of risks arising and then of course in the different kinds of safeguards to be adopted. In a reference to breeder reactors it cites the usual "dangers" arising from their preferred fuel being "plutonium of weapons grade" while appearing to underplay the contribution that any grade of plutonium can make for the same purposes or the relative attractiveness of enriched uranium for bomb making. In general, one would have preferred SIPRI to examine various aspects of the plutonium and breeder issues in a perspective which would inform and admit a reader's own judgment to be made rather than appearing to lean towards an anti-breeder if not an anti-nuclear viewpoint. For example, does SIPRI have a view on whether proliferation stemming from breeders can be forestalled by proper design, both technical and institutional; if it can, should proliferation-resistant breeder systems be set aside in face of forecast world energy requirements and particularly in face of the forecast 'CO₂ problem' arising from the burning of fossil fuels?

From the point of view of avoiding unnecessary alarm, or providing the neutral reader with a clear perspective, the section on 'Accidents of nuclear weapon systems' is one of the most unsatisfactory in the Yearbook. Whether the picture is presented as "substantially more serious accidents involving US nuclear weapons than the public were aware

World military expenditure, 1957-76



Source: SIPRI data

of" or "about one every two and a half months", the text on these is too often laced with such expressions as "it is reasonable to assume . . ."; "it is not unlikely"; "there is thus even some possibility . . .". These are the phrases which may leave some readers with an impression of frequent nuclear accidents when only a related weapons 'system' or vehicle may have been involved in either a major or minor accident, or even only an incident of some kind, and where "there is no record of any of them having involved nuclear weapons in transport" or where "there is no indication of whether or not any of these accidents involved nuclear weapons".

The text specifically states that "there is still no direct evidence of involvement of nuclear weapons in the case of the French and British weapons systems", yet the same chapter contains a table under the heading of "British nuclear weapon incidents". Admittedly this particular inclusion is explained away in a series of footnotes but, even within SIPRI's own justification for such presentation, would it not have been more helpful and reassuring to the reader to make the point that in the West there is no chance of a nuclear accident, involving even the slightest escape of radioactivity, being concealed, and that on this basis the record is one pointing to the very high standards of safety employed? In any event, in the light of the evidence presented, it is difficult to accept what the conclusions offer as being "clear" or "incontestable fact".

These various observations in respect of SIPRI's presentation of both non-nuclear and nuclear issues are made to illustrate the need for careful examination of various assumptions made in SIPRI's analysis before accepting the general conclusions drawn. At the same time they illustrate once again the dangers about which *Nature* editorials have warned in previous years and, hopefully, will encourage SIPRI to avoid a continuing trend that could so easily destroy the value of a reliable and objective reference work for which the need is great. □

Man-made process

Chris Sherwell reports on a study of man's involvement in the spreading desert

HOWEVER discomfiting it is for Western man to learn that he wastefully squanders his resources, his lesson is not nearly as painful as the one being learned by his contemporary living in the arid zones of the underdeveloped world. For there, that most precious and finite of resources, the land itself, is actually being lost, and at an alarming rate.

Some wealthy countries experience the same problem. Fifty million of the 163 million acres managed by the United States Bureau of Land Management, for example, are in 'poor' or 'bad' condition. But the US has the wherewithal to combat the phenomenon: the causes of desertification are understood imperfectly, but well enough for something to be done.

The real problem is conjuring out of nothing the political will to implement a comprehensive plan that deals with the poor countries' marginal lands. It is a problem which will confront the massive UN Conference on Desertification starting at the end of this month in Nairobi, when just such a plan will be drawn up. As with past jamborees of this sort—on Water earlier this year, Human Settlements last year or the Environment in 1972—a flood of papers is besieging the conference. One of the more important was published last weekend by the Washington-based Worldwatch Institute*.

Entitled *Spreading Deserts—The Hand of Man*, it is, as its title suggests, unequivocal about causes and solutions. It calls desertification an ecological phenomenon but says it is a human problem. "People cause it, people suffer its consequences, and only people can reverse it." Governments may find it difficult to devote substantial resources to combating a "seemingly long term and nearly invisible problem like ecological deterioration", but the consequences of desertification—undernutrition and famine, unemployment and migration, deepening poverty and human desperation—are "neither distant nor invisible".

Some of the statistics cited in the study confirm the need for action. About 14% of the world's population—630 million people—live on arid or semi-arid lands. Of this number, some 12% live on lands already almost useless and face the loss of their livelihood.

The decline in the quality of the land has meant a decline in the quality of people's diet, chronic ill-health, swollen ranks of urban migrants and lost economic potential. Indeed, cumulative degradation of rangelands and non-irrigated farmlands in the arid zones has, according to the study, held their combined annual productivity "more than \$12 billion below its potential level"; of sixteen underdeveloped countries in the arid areas, for example, only two significantly increased their per capita grain output between 1950 and 1975.

This decline is more popularly portrayed in another way. Along the southern fringe of the Sahara alone, 650,000 km² of land once suitable for agriculture and grazing has been lost in the past 50 years. Some 100,000 ha of range and cropland are lost to desert each year along the Sahara's northern margins. Elsewhere—in the Middle East and in Western Asia—the process's hold is "particularly long-lived and advanced". It is also taking place, as the accompanying map shows, in many of the western hemisphere's drier regions.

However, although natural desert encroachment is a real enough threat in some areas, the process of desertification is more correctly one where human action causes the desert to spread. A product of land-use patterns as well as climatic fluctuations, desertification will occur where land is abused in arid and semi-arid zones regardless of the actual proximity of the climati-

cally-created deserts. The effects of severe and prolonged land abuse are, of course, intensified by extended droughts. But to review the current theories and knowledge on world climate, says the study, is not only to "sense the urgent need for research efforts" on climatic trends; it is also to "see without question that people and their livestock are helping to downgrade the carrying capacity of arid lands and to create new spots of desert".

Desertification is not new; what makes today's problem especially acute, says the study, is the scale of suffering when the rains fail, and the scale of destructive human pressures. Rudimentary modern medicine has helped produce the 3%-a-year increase in population prevailing in the areas, which means more people are reliant upon the pastures and croplands there. At the same time the modern political and social order has grown inimical to the existence of the desert dweller, restricting nomadic groups who are well-adjusted to a harsh environment and encouraging sedentary agriculture.

The results are all too obvious. More people with more herds cultivate more areas to feed more mouths. Soils deteriorate, yields decline, pastures disappear, grazing land grows more distant and land around more intensively used water holes perishes. People become a threat to themselves: what they do to ensure individual survival in the short term is detrimental to the society's long term survival. As the Worldwatch study sees it, social systems and ecosystems are on a collision course; populations in the arid areas have reached the ecological danger point.

The study is also clear about what needs to be done. For a start, herd

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*Eric Eckholm and Lester R. Brown, *Spreading Deserts—The Hand of Man* (Worldwatch Paper 13, Worldwatch Institute, Washington; August 1977).

sizes should be reduced. Livestock should be valued for its quality rather than its abundance. If animal numbers in the Sahel were maintained at half the 1971 level, with modern management techniques the region's output of meat and milk could be double that level. Farming in the sedentary zones should also be improved, to halt its spread onto pasturelands. Farmers need knowledge and equipment to grow sufficient food using the best lands without loss of fertility; back-up for subsistence farmers has hitherto been neglected in favour of cash crops, which have themselves exacerbated the lot of the rural poor.

In addition, couples must perceive the advantages of small families. Population growth, says the study, prohibits a return to the ecologically sustainable rotation systems, so new cropping systems, along with tree-planting programmes, are the only alternative. Also, to urge the resettling of nomads and the killing of goats is to oversimplify: a modernised version of the nomadic way of life may be necessary, with clan leaders, aided by specialised advice, regulating grazing and migration through large herding cooperatives.

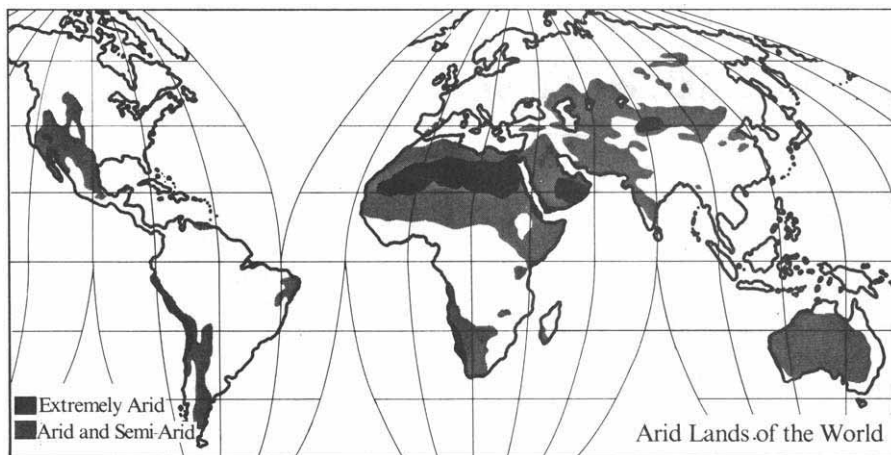
A major goal of arid lands development, says the study, must be to prepare peoples and economies to live through the inevitable droughts without trauma. Protection against famine, it says, is not a simple technological problem. Its success depends on nothing less than "the broad shape of national development and the character of the prevailing socio-economic institutions".

Plainly the challenge is great. It is a challenge facing the UN Conference. If that fails, and past experience does not augur well, the people most affected will be left to face it alone. They have done so before. But their chances of successfully continuing to do so are not improving. □



Sudan scene

UNESCO map by P. Meigs



ICARDA gets down to business

THE International Centre for Agricultural Research in the Dry Areas (ICARDA), having shaken off its troubled origins, is poised to embark on an ambitious research programme under the guiding hand of Dr Harry S. Darling, now president of Wye College, who is preparing to move to Beirut as ICARDA's director general.

The most recent of the international agricultural research centres, ICARDA will focus on improving food production in areas with an annual precipitation of 300–800mm that is concentrated largely in the winter months. Its findings will affect a region extending from Morocco to Pakistan with crops covering some 70 million hectares in 20 countries.

ICARDA staff members like to say that the centre "was born running", because it absorbed some of the facilities and personnel of the Arid Lands Agricultural Development (ALAD) programme of the Ford Foundation. But it was soon faced with obstacles that brought it almost to a complete halt. After the decision to establish head offices in Beirut in Spring 1974, war broke out, and most of the staff scattered to Cairo, Aleppo and Damascus. Negotiations with Syria and Iran to obtain land for research stations were conducted chiefly by Canada's International Development Research Centre (IDRC), which had been appointed the executing agency for ICARDA; formal agreements were signed with Syria in June and with Iran in July last year. IDRC also reached an agreement with the Ford Foundation to insure that ALAD activities relevant to ICARDA's mandate, notably the collection, testing and exchange of plant genetic materials, were continued.

On top of its research on the region's crops, ICARDA has been designated as the world international centre for barley, lentils, and broad beans. Barley, one of the world's oldest crops, is particularly important in the region. But wheat is usually preferred because it has a higher gluten content and makes a more cohesive loaf of bread. Barley's two major advantages over wheat, however, should encourage its utilisation as human as well as animal food. One of these is that in regions with less than 500mm of annual rainfall it can outperform wheat; where rainfall is 375 mm or less, it is practically the only cultivable cereal. The other advantage, hinted at by current research, is that barley is susceptible to improvement, notably by an increase in content

of lysine, an essential amino acid that is absent or exists only in minimal amounts in most food grains.

At present, barley yields in the Middle East are very low, and four ALAD-ICARDA barley varieties introduced in nurseries in 1976 indicate that performances can be considerably improved. Research is under way to deal with problems of disease susceptibility, lack of winter hardiness, intolerance to high temperatures, and insects. These trials will be undertaken in the ICARDA stations near Aleppo and Tabriz.

The food-legume crops improvement programme also continued in 1976. It consisted mainly in breeding and distributing germ plasma of three major pulses: lentils, broad beans and chickpeas. The major emphasis is on the development of high-yield, stable varieties, and of lines suited to mechanised harvesting. There is a forage-crop improvement programme, and some of the ALAD research work in wheat, maize and grain sorghum is being continued; so is the evaluation of several hundred lines of triticale, a man-made hybrid of wheat and rye.

Next year, ICARDA is expected to expand its activities. These will include the study of environmental systems throughout the region, the establishment of principles on which to base the development of appropriate farming strategies, and research into socio-economic constraints that limit the actual and potential production of existing farming systems. Finally, a training programme to help apply research findings is being set up.

From its inception until the end of last year, ICARDA's total budget barely exceeded \$3 million. But now that 'running speed' is being resumed and total staffing has exceeded 50, more funds are required for operations and capital development. Interest in the programme is illustrated by the commitment for 1977 of a total of \$4 million, the major contributors being the USA with \$1 million, the IDRC and Canada with \$600,000, Iran and Saudi Arabia with \$500,000 each, and the World Bank and Australia with \$220,000 each. Estimated expenditures of \$4.85 million for 1978 and \$7.77 million for 1979 have been submitted to the Board of Trustees, which is made up of representatives of some of the region's governments, agricultural research centres and donor agencies.

Alexander Dorozynski

USA

Pieces of action

Sandy Grimwade reports from Washington on some of the non-legislative developments concerning recombinant DNA research

ALTHOUGH much of the attention of the followers of the recombinant DNA debate has focused on the progress of the controlling legislation at present under consideration in Congress, activity in research labs and on campuses has not been completely stilled. A major supplier of laboratory chemicals has taken some unprecedented steps to control the use of its restriction enzymes—the enzymes which are used in the preparation of recombinant DNA molecules. A Maryland lawyer has sued the National Institutes of Health (NIH) on behalf of his son to postpone recombinant DNA experiments which he claims are at present illegal. And another group of scientists has petitioned Congress over the proposed legislation.

The firm which has taken a step practically guaranteed to lose it customers while bringing praise from those who favour a cautious approach to recombinant DNA research is Miles Laboratories Inc. Miles has been an important supplier of restriction enzymes for several years, and last year sponsored a symposium on recombinant DNA research. In themselves restriction enzymes can cause no hazard; a competent molecular biologist, moreover, would be able to prepare a private supply with a week or so of work. Miles now requires its customers to appoint a control officer who will be responsible for ensuring that its reagents are used in accordance with the NIH guidelines, and who must sign all orders for such reagents.

A spokesman for Miles said that the firm had taken this step on both moral and legal grounds, and that the restriction will be kept in force until the federal legislation now pending becomes law. Miles is applying this condition to all sales of restriction enzymes on the advice of its lawyers so that the entire burden of responsibility for their use is shifted to the customers. The spokesman admitted that the reaction from scientists to this additional bureaucratic requirement had not been entirely favourable. Its action is unilateral; other companies in the business are apparently not planning to follow suit.

The action of the attorney in Frederick, Maryland, who filed suit

under the National Environmental Protection Act on behalf of his 2-year-old son against NIH, was aimed at halting experiments planned to take place at Fort Detrick before publication of an Environmental Impact Statement (EIS). The experiments, which would involve the splicing of segments of the mouse polyoma virus genome as well as the whole genome into plasmids and cloning in *E. coli*, would under the NIH guidelines require the highest level of physical containment, the P4 level. The objective of the experiments is to discover whether the cloned gene segments of the polyoma virus, which causes cancer in mice but has no effect in humans, are expressed in *E. coli* and whether the gene products can raise antibodies in mice treated with the *E. coli* clones.

NIH have agreed to produce an EIS in 30 days, although it is not clear at this point whether the statement will address the potential impact of this

particular experiment or recombinant DNA experimentation as a whole. The attorney, Ferdinand Mack, sees the NIH action as a concession that they were ignoring the law requiring the publication of an EIS. In fact the EIS from NIH on the complete recombinant DNA guidelines has been hanging fire for over a year now, a point which does not follow the letter of the National Environment Protection Act. Friends of the Earth have filed a separate injunction enjoining a halt to all federally funded research in this area until the EIS is published.

The latest petition from scientists follows the statement about a month ago from the Gordon Conference on Nucleic Acids urging Congress to drop or tone down the proposed legislation. Signed by 140 scientists attending the Conference on Biological Regulatory Mechanisms—85% of those attending—the new statement was sent to members of Congress as part of a late-starting but vigorous effort by the proponents of recombinant DNA research to urge more lenient legislation. □

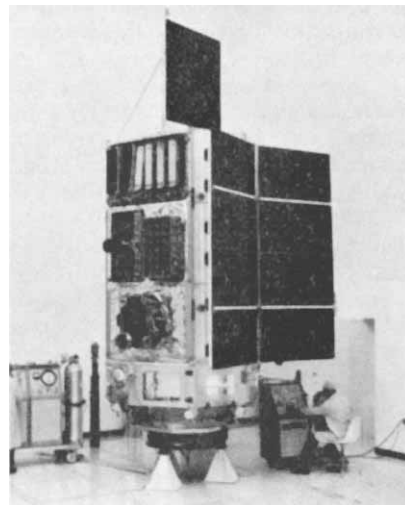
Scanning the universe

X-RAY astronomers are not having much luck at the moment. ESA's decision to schedule Exosat on the Ariane launcher upset the European space community, less because of Ariane's unknown reliability than because of the spectre of budget cuts for other projects and an almost certain delayed start to the mission. Though US astronomers have fared no better in recent months, some improvement may now come with the first satellite of the HEAO mission, which NASA expects to launch this week.

The High Energy Astronomy Observatory (HEAO) project was initiated in 1968. Budget cuts have since halved its size, but still the three-satellite mission involves the heaviest Earth-orbiting satellites ever launched. NASA had hoped to launch the first satellite, HEAO-A, on 15 April, but problems with the spacecraft's gyroscopes caused cancellation at the last moment; re-design and qualification of the gyros have now been completed. Though a launch scheduled for 30 June was missed, lift-off is fixed for the early morning of 12 August.

The HEAO observatories are designed as sensitive high-resolution detectors of X-rays and γ -rays. Building on the success and experience of such missions as Uhuru, Copernicus, Ariel V and SAS-3, HEAO could help resolve controversies over the existence of black holes and the nature of quasars, pulsars and X-ray bursters. No one commits \$237 million solely to fashion, however, and the HEAO mission has a solid, professional set of scientific objectives to pursue.

HEAO-A is an all-sky X-ray survey mission. A circular, 445-km high orbit will place it above the absorbing atmosphere and below the damaging radiation belts. During its six-month lifetime it will scan the complete sky and systematically



map both discrete X-ray sources and diffuse X-ray emission and absorption. The four experiments on-board provide high resolution (up to 5 arc-seconds) for position and size determinations and good time resolution for investigating the complex behaviour of variable sources. Crude spectral data will also be obtained.

HEAO-B is due for launch in 1978. Containing a wide-aperture imaging X-ray telescope with a sensitivity 10,000 times greater than any previously flown, it will latch on to specific sources of interest pinned down by HEAO-A and study them in great detail. The mission lifetime is estimated as one year. HEAO-C, another all-sky survey satellite and due for launch in 1979, will systematically scan for cosmic and γ -ray sources.

Stuart Sharrock

USSR

Well provisioned

The Soviet Union is at present going through the rituals of adopting a new constitution. Vera Rich reports

THE DRAFT of the constitution which has been presented to the public and is now the subject of 'discussion' (in effect, eulogy and explanation) in the media is, from a political point of view (State structure, the rights and duties of citizens and so on), little different from the existing version. But there is one interesting feature: the emphasis placed on the State's involvement in Science.

The existing constitution, promulgated in 1936, makes virtually no mention of science. In contrast, the new draft stresses that "in accordance with the requirements of society, the State guarantees the planned development of science and the training of scientific personnel", and the implementation of the results of scientific research in the economy (Art. 26).

A new clause (Art. 48) guarantees "freedom of scientific, technical, and artistic creativity", which is "ensured by the wide development of scientific research, inventive and rationalising activity and the development of art". The same clause pledges state support to "voluntary societies and creative unions", and guarantees the rights of

authors and inventors. The "right to work" is now extended (Art. 40) to include the right to choose a profession "in accordance with vocation, capabilities, professional training, education, and in accordance with State needs".

A somewhat ambiguous reference to science in the 1936 version has now been clarified: instead of guaranteeing the right of asylum to foreign citizens persecuted for "defending the interests of the working people, or for scientific activities", which suggested that the Soviet Union hoped to play host to latterday Galileos, the reference is now to "progressive social, political, scientific, or some other creative activity".

The new references to science clearly indicate a serious government commitment. To stress their importance, *Pravda* recently published a long statement by scientists of the Siberian Branch of the Soviet Academy of Sciences, a body which, being less than 20 years old, can well serve to express the viewpoint of the modern scientific community. In effect, the statement proved to be little more than a eulogy of the Soviet system of "guaranteed employment".

Not surprisingly, the eulogies are not echoed by dissident and *refusnik* opinion. Dr Veniamin Fain, a physicist who was allowed to emigrate in June,

recently told *Nature* that he was very doubtful that the new clauses would bring about any major changes as regards academic freedom, and the status of *refusnik* scientists.

The picture he describes, of scientists dismissed from their posts and forced to find work as janitors or lift-attendants simply because they wish to leave the country, accords ill with the new pledge of choice of profession in accordance with one's education. And the history of the Voronel-Azbel seminar hardly reflects the "support" pledged to "voluntary [scientific] societies". Nevertheless, a right once granted on paper can form an important rallying point for those who wish to press for its implementation. □

ARGENTINA

Scientist's plight

ELENA H. SEVILLA is the latest Argentinian scientist whose plight has come to public attention. Senora Sevilla is a 29-year-old atomic physicist who had been teaching at the University of the South in Bariloche and doing research at the Balseiro Institute.

She was arrested in November 1975, just five days after having had a Caesarian operation and whilst still in hospital. Her husband had been arrested two months earlier. Lack of evidence against her led to her acquittal in a court in Rawson in January 1976, but since then she has been held in preventive detention "at the disposal of the executive power". She has been moved several times from prison to prison, most recently to the Villa Devoto in Buenos Aires. There she shares a cell with 20 other women in conditions which are by no means good. Diet causes great concern, and prisoners, whilst allowed to receive three books each month, may not have university textbooks or books related to economics, history, chemistry, physics or languages.

In December 1976, Senora Sevilla applied for permission to leave the country under Article 23 of the Constitution, which allows for those held in preventive detention without charge or trial to opt for exile if they so wish. She has never received a reply. According to Amnesty International, Elena Sevilla has never been politically active. Amnesty asks fellow scientists to write on her behalf to the President, General Jorge Rafael Videla, Casa Rosada, Buenos Aires, with a copy to the local Argentinian Embassy.

EEC

Halocarbon decision

The European Commission has taken a first cautious and hesitant step towards restricting the use of halocarbons in EEC countries. A special correspondent reports

THOUGH not convinced that halocarbons are harmful, the European Commission is sufficiently impressed with the evidence so far, particularly from the USA, that it has decided to ask environment ministers in the Nine to ban any increase in manufacturing capacity for the halocarbons F-11 and F-12. The Commission also wants immediate measures to "encourage" industry to avoid any leaks of these gases, and to "encourage" industry to intensify research into possible substitutes and alternative methods. And it believes research into the effect of the gas on the environment should be stepped up, with more cooperation and coordination between researchers in the member states.

The Commission has been holding

meetings with national experts since January. If all goes according to plan, the EEC's Economic and Social Committee and the European Parliament will give their views on the Commission's proposals in September and October, and the environment ministers will make their decision in November. The Commission will then in the second half of next year assess the effects of halocarbons on man and the environment and draw up an EEC policy on the subject. Such a policy would need the approval of the ministers of the member countries.

In the meantime the Commission will see if it should try to co-ordinate the work, or if perhaps it should give limited aid under its programme of indirect action. European research at present is concentrated on measuring, on Earth and *in situ*, concentrations of the minor constituents of the atmosphere, studies of spectral properties and reaction speeds and development of mathematical models. □

correspondence

MLS international standard

SIR,—It is with regret that I report an error in the article 'Astronomy's own cloud' (9 June, page 478). The Australian system, 'Interscan', one of several Microwave Landing System (MLS) proposals, has not been adopted as the next international standard, and has not (yet) beaten all comers. The matter is, sadly, complicated if not bedevilled by partisan politics and if you were to read certain American reports, for example *Microwave Systems News* (June 1977, page 33) you would learn: "US Landing System going world wide".

The pedantic, boring truth is that the All Weather Operations Panel of the International Civil Aviation Organisation (ICAO) met in Montreal last March to consider the various MLS proposals. They recommended by a narrow majority vote (6-4), that the 'scanning-beam' (US-Australian) MLS scheme be submitted to the ICAO Air Navigation Commission for consideration for adoption as the international standard at an ICAO meeting to be called later this or early next year. Recommendation for adoption—no more, no less.

It is not altogether clear from the generally accessible published material just what the differences between the American system and the Australian 'Interscan' are. We can certainly say that the Australian idea was taken over by the Americans, whose own originally proposed schemes were demonstrated to be less than competitive. In this sense the Australians do indeed have cause for pride.

One must not forget that the Germans and the English both have advanced MLS proposals, and that the selection at Montreal of the US-Australian scheme was perhaps helped more by procedural niceties than by an honourable and convincing demonstration of the inferiority of the European work (*Aviation Week and Space Technology*, 28 March, page 26 et seq., *Frankfurter Allgemeine Zeitung*, 14 April, page 8). This was the subject of objections at the time and the reason for protest abstentions in various votes during the meeting.

One cannot altogether blame the Australians for blowing their own trumpet since they have suffered sorely in publicity at the expense of the Americans—their trumpet should

nevertheless play the written notes and not extemporise.

It is to be expected that the European interests in MLS will pursue vigorously a re-appraisal of the Montreal deliberations, with a view to demonstrating the technical superiority of one of the systems over the others—a demonstration which has not yet been attempted.

Yours faithfully,

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Wind power for the UK

SIR,—Dr Clement states that my estimates of wind-power are "grossly exaggerated" and that the environmental impact would be unacceptable. He compares my results with those of the recent Department of Energy report *The prospects for the generation of electricity from wind-energy in the United Kingdom* which considers the installation of windmills on hill-top sites (many of which would undoubtedly be environmentally unacceptable besides involving greater construction and transmission costs) and within a 15 km coastal belt. The total area involved in deriving their "upper limit on energy" is confined to about 1% of the UK land area. The more dispersed system which I discussed covers—as Dr Clement correctly computes—some 5×10^4 km² of land area, reduces the transmission costs per kW, and provides 20 times the energy.

The impact of wind generators on other land uses such as agriculture is minimal, and the visual impact must be judged in relation to that associated with any other scheme capable of providing comparable useful energy. If, for example, it were proposed to provide the UK energy gap predicted by the end of the century by the construction of new nuclear capacity on coastal sites, much of the south and east coasts of the country would require an average density of nuclear stations corresponding to about 0.2 GW km⁻¹ of coastline. If, as in my wind example, environmental and other objections permit only about one-third of the coastline to be so used, these areas will require about one 275 kV twin-wire transmission line running inland for every 2 km of coastline. At 4 towers

per km the density of pylons (whose construction is very similar to that of the windmills I considered) is then about twice that of the windmills. The nuclear stations themselves would of course involve both visual and other environmental problems as well.

Dr Clement seems to prefer to ignore the energy problems which we, and other countries, now face; perhaps he believes—as the Secretary of State has recently stated—that 13 GW of additional nuclear capacity (half in FBRs) would be more than adequate to meet our energy needs "in the foreseeable future" (*Guardian*, 3 June). Although this capacity would replace the oil-fired generating stations now in use (11 GW), it could make little contribution to the much larger energy demand at present provided by oil and gas for transport, industry and the heating of buildings. Owing to the large fluctuations in the latter, an installed capacity of 200-250 GW would be needed by the end of the century.

A nuclear capacity of 13 GW may indeed represent what it is feasible to build on this time scale, and if so it is clearly essential both to introduce every possible economy measure now and to proceed as fast as possible with the development and installation of alternative sources capable of making a more significant contribution. Wind-power is the most highly developed, has a large potential and is the cheapest, although wave, new forms of tidal and direct solar energy may also be important.

In comparing the cost of wind energy with that derived from nuclear and fossil fuel stations the Department of Energy report has considered only the replacement of base-load electricity, rather than the much more important energy gap discussed above. Owing to the large fluctuations in this energy demand, it is not permissible, as the report has done, to include the interest on capital for wind generators but exclude it for conventional systems, and the high costs they derive for wind-power are not relevant; the actual costs, both capital and running, for producing a given useful energy from wind are about one-third those of a nuclear system.

Yours faithfully,

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news and views

Enzymoimmunoassay: techniques and uses

from J. Landon

IMMUNOASSAYS are being used increasingly in clinical practice to detect and measure antigen concentrations in biological fluids. Some procedures do not employ a labelled reactant, including radial immunodiffusion, rocket immunoelectrophoresis and various automated systems. They are, however, applicable only to proteins, and although simple and precise have limited sensitivity and need high concentrations of monospecific antisera. In 1960 Yalow and Berson introduced a radioimmunoassay (RIA), to determine circulating insulin levels, using ^{125}I -labelled insulin and appropriate antiserum. Use of a radioisotope for labelling a reactant overcame the need for monospecific antisera, improved sensitivity markedly and enabled haptens as well as proteins to be assayed.

Many other possible labels have been studied in recent years, including enzymes, fluorescent or chemiluminescent molecules, viruses, proteins, particles and free radicals. Enzyme labelling has been the subject of most work and provides a feasible alternative to radioimmunoassay. Various names are current, including enzymoimmunoassay (EIA), enzyme-linked immunoassay and enzyme-linked immunosorbent assay (ELISA). The term EIA is preferred because of its similarity to the term radioimmunoassay and because it prevents confusion with the measurement of enzymes by radioimmunoassay.

Advantages and disadvantages

Reasons for the emphasis given to enzyme labelling include its previous applications in histochemistry and cytochemistry; the availability and relative cheapness of many enzymes and of manual and automated systems for their assay; the long shelf life of the labelled product (which can be measured in years); lack of hazards during labelling; and a sensitivity, specificity and applicability similar to

radioimmunoassay. Nonetheless, the additional step of determining enzyme activity prolongs the assay and increases imprecision, while the amplification made possible by the use of an enzyme to attain sensitivity will also magnify any error. Furthermore, biological samples may themselves contain enzymes with similar biological effects which can cause a high and variable background, and other factors such as haemolysis may affect the result.

The ultimate success of EIA will depend on its merits as compared with the widely used radioimmunoassay. The relative merits of radioisotopes and of enzymes for labelling has recently been surveyed by Ekins (*Lancet* ii 176; 1976). Briefly, RIA is relatively inexpensive, very sensitive and precise, and the sample provides no background signal. But isotopically-labelled reactants have some disadvantages including a slight health hazard associated with their production, and a limited shelf life. Short shelf life poses considerable commercial problems since it necessitates frequent radioiodination and increases costs of production, quality control and distribution.

Analytical techniques

Various procedures using enzyme-labelled antigen or antibody to assay antigens have been developed. The reactant is labelled with one of a wide variety of suitable enzymes (acetylcholinesterase, alkaline phosphatase, malate dehydrogenase, peroxidase and β -galactosidase, for example) (see Sharpé *et al. Clin. Chem.* 22, 733, 1976; Wisdom *Clin. Chem.* 22, 1243; 1976). At the end of the assay the enzyme-labelled reactant is easily measured by reaction with substrate.

Techniques using labelled antigens

One approach involves a separation step and is similar to RIA; enzyme-labelled antigen and the sample are incubated with a limited amount of specific antibody and the proportion of

label distributed between antibody-bound and free fractions gives, by comparison with a set of standards, the concentration of antigen in the initial sample. Antibody-bound and free fractions may be separated by adding a second antibody, or by the use of solid-phase systems in which the first antibody is linked to an insoluble matrix. A combination of these two methods, in which the second antibody is covalently coupled to cellulose—the double antibody solid phase system (DASP)—has been successful.

The separation step in an EIA can serve a second important function, namely to separate the exogenous enzyme being used as the label from any endogenous enzymes in the sample with similar biological effects or from any other constituents in the sample, such as enzyme inhibitors, which could influence the signal.

The first EIA was developed for immunoglobulin E by Engvall and her colleagues (*Immunochemistry* 8, 871; 1971) and assays have now been described for a wide range of antigens including albumin, carcinoembryonic antigen, human placental lactogen, insulin, cortisol, total oestrogens and testosterone.

The separation step can be avoided in some EIA. In one category, which can be termed 'quenching' assays, there is steric hindrance by the antibody in the bound fraction of the reaction between the substrate and active site of the enzyme (Rubenstein *et al. Biochem. biophys. Res. Commun.* 47, 873; 1976). Thus only the free fraction retains enzyme activity. Use of enzymes such as malate dehydrogenase and glucose-6-phosphate dehydrogenase that require cofactors, obviously enhances the potential for steric hindrance. Assays of this type have been developed for various haptens, including several drugs of abuse, anti-epileptic drugs and digoxin and have been applied to urine, saliva and plasma.

Alternatively, assays exist of the

'enhancement' type in which antibody binding potentiates enzyme activity. Such an assay has been described for thyroxine using a thyroxine derivative covalently linked to malate dehydrogenase such that the enzyme has little activity. Enzyme activity increases markedly after adding anti-thyroxine antibodies (Ullman *et al. Clin. Chem.* **21**, 1011; 1975). This assay has also been incorporated onto a multi-channel biochemical profiling instrument (Galen & Forman *Clin. Chem.* **23**, 119; 1977).

The time saved and the precision gained by avoiding separation compensates for the enzyme detection step and such assays are being increasingly used. This approach is, however, probably not applicable directly to the assay of proteins, and problems due to comparable enzymes and other constituents in the sample may limit the sensitivity that can be achieved.

Techniques using labelled antibody

The standard approach is to react the target antigen with excess labelled specific antibody and to remove unreacted labelled antibody by excess antigen covalently linked to a solid phase so that the enzyme activity associated with the antigen in solution is directly related to the antigen concentration (Maiolini *et al. J. Immun. Methods* **6**, 355; 1975; Barbour *J. Immun. Methods* **11**, 15; 1976).

Sandwich technique: this approach was first introduced by Wide in 1971, using radioisotopic labelling, and is applicable to the assay of large molecules with more than one antigenic determinant. It is designed to improve

specificity by excluding fragments of the molecule from influencing the results. Excess antibody covalently linked to a solid phase and directed against one end of the antigen is incubated with the sample and, after suitable washes, excess of a second, enzyme-labelled antibody directed against the other end is added. After further washes, the enzyme activity is determined. This provides a direct measure of the amount of antigen present and has been applied to the assay of both rat and human alpha foetoprotein (Maiolini & Masseyeff *J. Immun. Methods* **8**, 223; 1975).

A variation of the above is to add an excess of enzyme-labelled second antibody directed against the first. This has the merit that, for example, an enzyme-labelled goat anti-rabbit gamma globulin sera can be used for all assays based on rabbit first antibody.

In conclusion, it seems unlikely that the use of isotopically-labelled reactants will be replaced in the near future for assays requiring extreme precision or sensitivity in the pmol l^{-1} range. In our experience EIA has proved somewhat more complex, taken longer to perform and has been slightly less precise than RIA. However, EIA and related techniques have an important part to play in the developing world, in countries with severe restrictions on the use of radioisotopes; for such yes/no cases as the diagnosis of pregnancy or detection of Australia antigen (a marker of past or current infection with hepatitis) and, finally, for hapten assays in which their use avoids the need for a separation step. □

It is generally accepted that Hercules X-1 is a neutron star which has a very strong magnetic field. A field strength of around 10^{12} gauss, similar to those deduced for radio pulsars, is anticipated. The X-ray luminosity is produced by material, stripped off the surface layers of HZ Herculis, being accreted by the neutron star. A neutron star (radius about 10 km) is so compact that material falling onto it releases about 10–20% of its rest mass energy when it strikes the stellar surface. Thus a small trickle of material can give rise to a considerable luminosity. Material accreting onto the star is highly ionised and is therefore channelled along the stellar field lines down onto the magnetic polar caps. Thus the accretion energy is liberated in two hot spots, each about a kilometre in diameter, on the stellar surface. Such a high luminosity radiated from such a small area can only be emitted as X radiation. Then if the magnetic axis and the rotation axis of the star do not coincide, a stellar rotation period of 1.24 s ensures that the emitted radiation is pulsed at the correct period.

Although these basic ideas have been around for some time, the details of the radiation processes have proved too complicated for reliable predictions of pulse shapes and pulse spectra to be made. There are many uncertainties concerning the emission of radiation at the polar caps, in particular because the radiation emitted by the material striking the surface interacts with the column of accreting matter on its way out. It is also unclear exactly where the accreting material releases most of its energy, whether at the base of the accretion column at the stellar surface or more gradually as the column settles slowly supported by radiation pressure. The velocity of the material that is emitting or interacting with radiation is also important since infall velocities can be as high as half the speed of light and relativistic effects can come into play. In such a strong magnetic field the electron orbits perpendicular to the field direction are quantised with levels separated by an energy $13 B_{12}$ keV, where B_{12} is the field strength in units of 10^{12} gauss. Thus quantum calculations of the emission and absorption processes have to be made for the role of cyclotron radiation in such strong magnetic fields.

A further complication has been introduced by a recent suggestion that the beam pattern of X-ray emission may be produced not so much by anisotropic processes occurring near the stellar surface, but rather by a shell of material at the Alfvén radius or the surface of the magnetosphere (of radius about a thousand stellar

The puzzles of Hercules

from James Pringle

ABOUT a dozen pulsing X-ray stars have now been discovered. Among these perhaps the most intriguing, and certainly one that has stimulated much research and headache is the star Hercules X-1. This has an X-ray luminosity of around $10^{37} \text{ erg s}^{-1}$ and the X radiation is pulsed with a period of 1.24 s. Almost as soon as this pulsing was discovered, the pulse period was found to vary in a sinusoidal manner with a period of 1.7 d. This, together with the fact that the X radiation from Hercules X-1 dis-

appears for 9 h once every 1.7 d, indicated that the pulsing X-ray star was in orbit around a companion star. The sinusoidal variation of the pulse period is due to Doppler shift of the intrinsic pulse period as the source orbits its companion, and the regular disappearance of the flux is due to eclipses. These ideas were confirmed by the discovery that the companion star was the already known variable star HZ Herculis. The side of HZ Her facing the X-ray star is so intensely heated by the X radiation, that as the binary star system revolves the apparent optical brightness of HZ Her varies by a factor of four with a period of 1.7 d.

radii) which has collected there before being channelled down to the magnetic poles. If the shell of material is not spherically symmetric—a likely event given the expected dipolar shape of the magnetosphere—then the variation in the amount of scattering or absorption of X rays in passing through the shell in the direction of Earth as the star and shell rotate can give rise to an apparent pulsed emission even if the neutron star produces a steady X-ray flux. In the absence of other information it was beginning to seem as if little progress could be made in the unravelling of these various effects.

Some recent observations, however, have given some cause for hope. At the Eighth Texas Conference held in Boston in December 1976 (see *News and Views* **265**, 402; 1977) J. Trümper announced evidence for a strong cyclotron emission line in the hard X-ray spectrum of Her X-1 in data obtained from a balloon-borne experiment. The apparent line is at 53 keV which if assumed to be cyclotron emission corresponds to a magnetic field strength of 4.6×10^{12} gauss. Because of background subtraction problems, this line although apparent in the raw spectral data, is more clearly visible when the data has been processed. The processing involves taking the spectrum of those photons received during the peak of the pulse, and subtracting the spectrum of the rest from it. In this way the background (assumed not to vary with a 1.24 s period) is removed. If the data is interpreted as for the most part a steady flux from the source, on which is superposed a fully modulated pulsed flux, then the spectrum obtained by this method is that of the pulsed flux. If this is not the correct interpretation then there are problems in deciding what the subtracted spectrum actually represents.

A paper by Coe *et al.* in this issue of *Nature* (page 508) reports confirmation of Trümper's results. The observations were made using the Ariel 5 satellite. Background subtraction is less of a problem with the satellite data, but even so the confirmed line seems to have shifted to 64 keV and the authors do not comment on the statistical significance of their result.

Nevertheless, the observations should provide a much needed clue to the X-ray emission processes occurring in Hercules X-1 and the other pulsed sources. Further observations—for example another balloon flight by Trümper's group this autumn with a larger area detector—are underway; thus not only do theorists have something to start thinking about but also the prospect, welcome or not, of a more rigorous test for any ideas they may come up with. □

Towards some enzyme mechanisms

from C. C. F. Blake

IN the heady days of the late 1960s, when X-ray diffraction began to reveal the structures of enzymes in atomic detail, it seemed that the comprehensive view of the active site and the binding of substrate analogues that the technique provided, would lead rapidly to the establishment of a mechanism for each enzyme that was studied. In rapid succession proposals for the catalytic mechanisms of lysozyme, chymotrypsin and carboxypeptidase were put forward that contained profound insights into the chemistry practised by hydrolytic enzymes. However, since that time, as attention has become focused largely on the intracellular metabolic enzymes, the regular reports of the structures of new enzymes have not been accompanied by the expected mechanistic breakthroughs. Not altogether unexpectedly, it is beginning to look as though the non-hydrolytic enzymes are not going to yield the secrets of their activity as readily as the hydrolytic enzymes. This rather disappointing picture has recently been relieved to some degree by proposals on the catalytic mechanisms of two 'second generation' proteases.

The first protease, thermolysin, is a member of the family of neutral endopeptidases that require zinc for activity. Following their work on the structure of the enzyme (Matthews *et al.* *J. biol. Chem.* **249**, 8030; 1974) which revealed that its polypeptide chain was organised in quite a different manner from the other known zinc-requiring protease, the exopeptidase carboxypeptidase, Matthews and his colleagues have now proposed that despite this difference the mechanism of thermolysin is very similar to that of carboxypeptidase (Weaver *et al.* *J. molec. Biol.* **113**, 119; 1977). The mechanistic proposals are largely based on an examination of the binding of the potent inhibitor Phosphoramidon (N-(α -rhamnopyranosyloxyhydroxy-phosphinyl)-L-leucyl-L-tryptophan). Phosphoramidon is located in the active site of thermolysin with one of its phosphinyl oxygens located 2 Å from the essential zinc ion, completing its tetrahedral environment, while the other oxygen hydrogen bonds to the carboxylate of Glu 143. The leucine residue of the inhibitor is located in a hydrophobic pocket that appears to represent the specificity site of the enzyme, accounting for its known preference for bulky aromatic residues on the amino side of the scissile bond. A

number of small conformational changes occur on binding but they are restricted to the active site region.

On the basis of the binding of Phosphoramidon, and some dipeptide inhibitors, Matthews concludes that the phosphinyl compound acts as an analogue of the presumed tetrahedral transition state intermediate of the enzymatic reaction. This has enabled a detailed model of the transition state of the true extended polypeptide substrate to be built into the active site, leading in turn to the proposed mechanism. The major features of the substrate binding are: the location of the carbonyl oxygen of the scissile bond as the fourth zinc ligand; the location of the large hydrophobic residue on the amino side of the scissile bond in the specificity site; and an extensive anti-parallel β -sheet interaction between the polypeptide chains of the bound substrate and the enzyme. On either side of the susceptible bond are a carboxyl group (Glu 143) which, together with an associated water molecule, is 'buried' by the substrate, and His 231 that forms a hydrogen-bonded pair with Asp 226, rather like the charge relay in the serine proteases. The proposed mechanism indicates that Glu 143 promotes the nucleophilic attack of the buried water molecule on the carbon of the polarised carbonyl group of the substrate, forming a tetrahedral intermediate, and concerted with this step, or following it, His 231 donates a proton to the nitrogen of the scissile bond, leading to its collapse. This mechanism is very similar to one of the two alternative mechanisms proposed by Lipscomb for carboxypeptidase (Lipscomb *et al.* *Brookhaven Symp. Biol.* **21**, 24; 1968), except that proton donation is carried out by the histidine in the 'charge relay' rather than a tyrosine residue. By reverse analogy, it can be argued that the thermolysin mechanism supports the proposal that Glu 270 promotes the attack of a water molecule on the substrate carbonyl in the carboxypeptidase mechanism, against the alternative of direct nucleophilic attack by the glutamic acid.

Precisely the same argument is used in the case of the second protease, penicillopepsin. The three-dimensional structure and amino acid sequence of this enzyme were reported recently (Hsu *et al.* *Nature* **266**, 140; 1977), clearly demonstrating it to be a member of the acid proteases; the last major family of proteases to be examined by X-ray diffraction. Now James and his

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colleagues (James *et al.* *Nature* **267**, 808; 1977) have immediately followed the description of the structure with a proposal for the enzyme's mechanism—just like the old days! This proposal is based largely on the remarkable environment of the two essential acid groups, Asp 32 and Asp 215, although binding studies have been carried out on the potent inhibitor 1,2,3-epoxy-3-(p-nitrophenoxy) propane (EPNP). The difference map of inhibitor binding shows that the two molecules of EPNP have reacted covalently with penicillopepsin, one with the carboxyl group of Asp 32, the other with the carboxyl group of Asp 215. Associated with inhibitor binding is clear evidence of a conformational change to Tyr 75 that brings it into the active site.

The key to the mechanism of the acid proteases seems to reside in the environments of the two acid groups. Asp 32 is located in an unusually tight hydrogen bonding net, that involves its carboxyl group in interactions with (1) the carboxyl of Asp 215, (2) the hydroxyl of Ser 35, (3) the main-chain NH of Gly 217, (4) a bound water molecule, and (5) the main chain atoms of the 32–33 and 33–34 peptide groups. This complex of interactions makes Asp 32 inaccessible for the formation of covalent interactions with substrates without conformational changes in the active site, which its particular structure seems to rule out. Asp 215 is on the other hand rather more accessible, but since it seems established that this group has the higher pK_a , it is unlikely to be the attacking nucleophile.

James points out that the resolution of this problem may be sought in the cluster of polar and ionic side chains that lie in the hydrophobic core near the two acid groups. These include Asp 304, which makes hydrogen-bond contact with the main chain carbonyl of Thr 216 (the same peptide whose NH hydrogen bonds to Asp 32), nearby is an ion pair, Lys 308 and Asp 11, which has as neighbours a buried tyrosine, residue 14, and a surface histidine, residue 57. With the exception of the histidine, all these residues are conserved in porcine pepsin. This cluster of buried residues is interpreted as an extended charge relay system. It is assumed that the binding of substrate in the large cleft of the enzyme will affect two associated hairpin loops carrying Asp 11 and Tyr 75, causing a minor conformational change to the former and a larger one to the latter, similar to that observed when EPNP reacts with the two acids, bringing Tyr 75 into the active site. The movement of Asp 11 would disrupt its ion pair interaction with Lys 308. A series of changes then ensues that involves Lys 308 pairing with Asp 304 and the pro-

tonation of the 216–217 peptide bond that would enhance the electrophilicity of the proton shared between Asp 32 and Asp 215. This proton could then function to polarise the carbonyl group of the substrate in a mechanism that is otherwise identical to the zinc-carbonyl mechanism of carboxypeptidase. Asp 32, the analogue of Glu 270 in carboxypeptidase, would promote the attack of its bound water molecule on the carbon atom of the polarised carbonyl group of the substrate, while Tyr 75, the analogue of Tyr 248 in carboxypeptidase, protonates the nitrogen of the scissile bond. Thus in James's view the mechanism of the acid proteases is essentially the same as that of carboxy-

peptidase, with modifications to make it competent at a low pH. This means replacing the Lewis acid, Zn^{2+} , with a proton whose electrophilicity is enhanced by the charge relay involving the protonated peptide, and the placing of Asp 32 in an unusual environment to keep it in the ionised state at very low pH.

Although the mechanism of the acid proteases proposed by James has some controversial features, not least the involvement of a peptide bond, the remarkable environment of the essential acid groups will provide a solid basis for any further discussion, and this is what the X-ray crystallography of enzymes should be all about. □

Visiting Halley's Comet

from David W. Hughes

COMETARY missions have been discussed in varying degrees of detail on both sides of the Atlantic for well over a decade; spacecraft have been chosen, experiments have been planned, data and science returns estimated, impact hazards evaluated. All this as yet to no avail. Why? Comet trips are not cheap; \$100,000,000 is a reasonable bargain-basement estimate. Also they represent a distinct new start in space exploration, needing new people and new ideas and the old teams of lunar, planetary and magnetospheric space scientists seem only too willing to plump conservatively for well tried experiments or at best minor advances in techniques. Also when the budget for space exploration is limited, the 'jobs for the boys' and 'let's play safe' syndromes come to the fore.

Comets are also minor objects in the Solar System. They are studied by a keen but small band of scientists. Very few of them get into the news, comet Kohoutek being the only one to make a stir recently and this disappointed most tax paying (and consequently space exploration supporting) people by being much fainter than originally predicted. The missions are also difficult; comets, being small, are hard to find and considerable launch and trajectory correction accuracy is required to get close to them. Ballistic transfer usually gives a fly-by speed of upwards of 7 km s^{-1} . Measuring the properties of a cometary coma and nucleus when you are flying by at $25,000 \text{ km h}^{-1}$ is not easy and also the time during which cometary pheno-

mena are being investigated makes up a very small percentage of the total mission time (however the same could easily have been said of the first Mariner trips to Mars and Ranger trips to the Moon).

How does comet Halley change all this? First, it has the advantage of being famous. Second, it has been around a long time. It passes perihelion about every 76 yr, is unusually bright and seldom goes unnoticed. Observations go back as far as 239 BC when it was mentioned in ancient Chinese records and it has been seen 28 times since. Third, it is rather awkward to get to, its retrograde orbit giving a ballistically transferred spacecraft a fly-by speed of almost 60 km s^{-1} . Halley is therefore an ideal candidate for a rendezvous mission, but this would require considerable expenditure of power. Ion drive and solar sail propulsion have been suggested as ways round this problem.

These two mechanisms are still at the theoretical stage but have caught the imagination of space technologists to such an extent that Halley's comet is being actively suggested as a first mission application for the two competing techniques. Scientifically, Halley's comet is as good a start as any to a programme of cometary investigation. The first mission must be regarded as the precursor to a series of investigations which will eventually take in comets of diverse qualities and build up a complete picture of the family. The exact form of the cometary nucleus is still a mystery and there is debate as to whether it is a condensed, dirty, icy snowball or a large swarm of smaller particles. Comets decay to produce meteor streams and so feed the zodiacal dust

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cloud. Cometary chemistry is very similar to that of interstellar gas but fortunately comets are closer. The interaction between the plasma in the cometary coma and the high speed magnetoplasma of the solar wind poses exciting physical problems. Comets are also probably in the form of the original condensate from the pre-Solar System nebula and as such furnish an outstanding clue in cosmogony.

Halley's comet was visible to the naked eye over a four month period at its 1910 apparition. The nuclear region was several thousand kilometres in diameter but on 18 May, 1910, when Halley transited the Sun, no solid nucleus was observed against the solar disk indicating that, if this existed, it must be less than 50 km across. The coma was about 3×10^6 km in diameter during the postperihelion phase, and had a continuous background spectrum with superimposed CN and C₂ lines. Transient phenomena were observed in the inner coma regions. Halley is a very dusty comet and also has non-gravitational effects acting on its nucleus leading to a lengthening of the orbital period by about 4.1 d per apparition.

The main scientific objectives of the fly-by are to image the nucleus, to find its size, shape, rotation period and colour, to determine the nature of the multiple condensations seen in 1910, to determine the abundance and spatial distribution of neutral molecules, radicals and charged particles in the coma and tail, to investigate the shockwave interaction between the cometary plasma and the solar wind, to measure the spatial density and size distribution of the emitted dust particles and to investigate the time variation of the coma's structure and brightness.

Opportunities for ballistic missions to Halley have been studied by Robert W. Farquhar and William H. Wooden II (NASA Goddard Space Flight Centre) and have been recently reported in NASA Publication No. X-580-77-46. At a fly-by speed of 60 km s⁻¹ the spacecraft will pass through the inner and outer coma in 14 and 18 min respectively. The miss distance from the nucleus would be about 2,000 km. The spacecraft could approach closer to the nucleus with onboard navigation but previous visual observations indicate that Halley has multiple nuclear condensations and it would be difficult to know which one to pick when the spacecraft is a considerable distance from the comet. At perihelion Halley is about 0.59 a.u. from the Sun. Perihelion passage occurs on 10 February, 1986 and most investigating spacecraft plan to visit Halley within a period of about 60 d either side of this date.

The Warsaw-Moscow soil structure project

from I. J. Smalley

A JOINT programme to provide a set of soil structure standards for use in engineering geology and soil mechanics has been under way for five years at the Universities of Warsaw and Moscow and is now nearing completion. The first applications of the scanning electron microscope (SEM) to soil structure studies were made in the late 1960s and in 1971 a project was initiated by B. Grabowska-Olszewska of the Warsaw University Engineering Geology Institute and V. Osipov and V. Sokolov of Moscow University with the aim of providing a complete range of reference structures from clay sediments of different ages and from different sedimentary environments.

The scanning electron microscope has not proved as effective a tool in the investigation of soil structures as was hoped when studies began and much of the earlier enthusiasm is now waning. There is, however, a growing emphasis in engineering geology and soil mechanics on the study of earth materials in some detail and Attewell and Farmer (*Principles of Engineering Geology*, Arnold, 1976) and J. K. Mitchell (*Fundamentals of Soil Behaviour*, Wiley, 1976) in their new books both present extensive sets of soil microstructures, but they are essentially illustrations; the relating of soil structure to engineering properties has not been achieved. A set of standard structures with related properties may help in the making of this necessary connection.

The Warsaw-Moscow project has been hampered by the lack of SEM facilities in Poland and the USSR and most of the structural photographs were obtained using the American

built 'Cwik-Scan' 106A SEM of Moscow University. Samples were taken from Poland and the European USSR and all geological areas are represented from Proterozoic to Holocene. The programme involved the examination and testing of 86 samples of eluvial, hydrothermal, marine, lacustrine, alluvial, glacial and glacial-lacustrine origins. Five structural elements were identified: primary clay particles, micro-aggregates, aggregates, grains and inclusions of microfauna, microflora and microcrystals of salts and ore minerals. The structural data, usually presented as 5000× and 500× photographs, is supplemented by a range of property measurements including specific gravity, water content, density of wet soil, porosity, specific surface, liquid limit, plastic limit, uni-axial and pure shear strengths and it is the relation of this set of mechanical data to the structural representations which makes the project so valuable.

E. M. Sergeev (Moscow) and W. C. Kowalsky (Warsaw) have editorial attachment and various western investigators have been involved; in particular David Krinsley of Arizona State University, doyen of the SEM sand gazers, has recently been in Warsaw helping with the final stages of the project; and R. Pusch of Lulea University has been giving encouragement. The 1973 Gothenburg Engineering Soil Structure Symposium organised by Pusch revealed the disorder and confusion in the field; perhaps the Warsaw-Moscow project will provide a necessary foundation for future studies.

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Rendezvous with Halley

Farquhar and Wooden put forward four possible missions. The first is a dual launch around 4 July, 1958 of the spacecraft using a single Space Shuttle, one planned to intercept Halley 63 d before perihelion (at 1.37 a.u.) the other 39 d after perihelion (at 1.00 a.u.). One approaches Halley from the sunward side the other from the anti-Sun direction. The second scenario again uses the Shuttle to launch two spacecraft, this time on 10 March, 1985. One intercepts Halley 58 d before perihelion passage, the other flies off to intercept two other comets, Giacobini-Zinner on 11 September, 1985 and

Borelly on 25 December, 1987 these interceptions taking place at 1.03 and 1.36 a.u. respectively. The third launches the spacecrafts at Halley in a trajectory that returns to Earth after passing the comet. They then swing past the Earth and are retargeted on Borelly to fly-by in January, 1988 or comet Tempel-2 in September, 1988. The final proposed mission has a dual launch in August, 1985, with both spacecraft encountering Halley at the same time, one passing the nucleus on the sunward side at a distance of about 2,000 km and the other moving through the tail about 30,000 km behind the nucleus.

All these ballistic missions would produce an enormous amount of fascinating new data concerning comets and they have the advantage of a late launch date so that the orbital parameter of the comet can be updated by observations taken as it comes towards the Sun. They also have a short transit time, the spacecraft usually encountering the comet about 8 months after launch. Launch energies are also relatively low, C_3 being around $12 \text{ km}^2 \text{ s}^{-2}$ for the missions that do not have an Earth return and about four to five times this for the one that does. One disadvantage is the high fly-by speed, most spacecraft encountering Halley nearly head on.

Solar sails and ion drive

Two rather unorthodox schemes have been proposed recently to get over this problem. Both require advanced propulsion capabilities and these capabilities have been little developed to date. Both require flight times around 3 to 4 yr and thus need launch dates in early 1982. Both schemes have the enormous advantage of enabling the spacecraft to rendezvous with the comet and move off with Halley on its journey away from the Sun.

J. L. Wright (Jet Propulsion Laboratories, California) and J. M. Warmke (Battelle Columbus Laboratories, Ohio) discuss the solar sail concept in the *American Institute of Aeronautics and Astronautics* paper number 76-808. Here a sail, between 300 and 800 m across, would be attached to an existing spacecraft, like Mariner or Pioneer, the propulsion force being derived from the reflection of solar photons. About $0.9 \times 10^{-5} \text{ N m}^{-2}$ could be obtained at 1 a.u. By tilting the sail with respect to the Sun's direction the spacecraft could tack on an inward or outward trajectory. No additional propellant is required; one literally sails through space. At 1 a.u. a typical spacecraft could be accelerated at between 0.2 and 1.3 mm s^{-2} , corresponding to a mass loading on the sail of between 40 and 60 g m^{-2} . Wright and Warmke suggest that with considerable development effort the sail could be ready for a first mission application in late 1981. $640,000 \text{ m}^2$ of sail could accelerate a 800 kg spacecraft to Halley. Launch would be around January, 1982 the vehicle first spiralling inward to about 0.3 a.u. then being cranked into an orbit of inclination 162° . Gaining energy it would move out to about 2 a.u. before returning to the inner Solar System nearly along Halley's orbit, rendezvousing with the comet after perihelion somewhere between 1 and 2 a.u. from the Sun.

The second scheme uses an advanced form of solar electron propulsion

and has been put forward by K. L. Atkins (Jet Propulsion Laboratories) and C. Terwilliger (Boeing Areospace Co.) in *AIAA Paper* 76-1069. They note the massive advance in electron propulsion performance. 1973-74 vintage required 50 kg of spacecraft systems to produce a kilowatt of power. New 3-mm thick solar cells and high powered, direct-drive ion engines have reduced the specific mass to 10 kg giving a fivefold increase in power to weight. To get to Halley the spacecraft would be launched around April, 1982 in an outbound flight towards Jupiter. The ion drive vehicle remains outside Halley's flight path, makes a powered 'U-turn' at about 4.5 a.u. and speeds back towards the Sun essentially already in Halley's orbit, simply waiting for Halley to overtake. Flight time is around 3.75 yr, rendezvous occurring about 50 d before perihelion and maximum cometary activity. The spacecraft then stays with Halley having in Atkins and Terwilliger's words "a pre-performance seat to watch the whole show".

Halley is even now coming in towards the Sun and has already passed the orbit of Uranus. It is hoped that we can be adventurous enough, both scientifically and financially, to get one of the schemes outlined above ready to meet it as it comes by in 1985-86: if not we will have to sit back until 2026. □

Environmental mutagenesis

from Margaret Fox

The 2nd International Conference on Environmental Mutagenesis was held at Edinburgh on 10-16 July, 1977.

A CENTRAL problem in the field of environmental mutagenesis is to design 'short-term' tests, using either prokaryotic or eukaryotic systems, or both, capable of detecting many different types of genetic damage. During the conference the deficiencies and advantages of the currently available tests were extensively discussed.

Carcinogens in most instances have produced positive results in screening programmes for mutagens in contrast to non-carcinogens (B. N. Ames *et al.*, University of California, Berkeley) and it seems likely that DNA damage is intimately involved in both cases. However, there are some notable ex-

ceptions to this rule. M. S. Legator (Department of Preventative Medicine, University of Texas) in discussing microbial activation systems such as Ames's *Salmonella* test, pointed out that not only do such systems fail to detect certain types of genetic damage, for example, chromosome non-disjunction, they also fail to detect several other classes of known mutagen and carcinogen such as ionising radiation, chlorinated pesticides, azo dyes and certain metal carcinogens.

The necessity for metabolic activation of many carcinogens is now well established, and S-9 preparations of rat liver microsomes are now routinely included in many screening tests. The almost exclusive use of liver preparations and the assumption that the inclusion of liver microsomes in microbial systems will accurately mimic *in vivo* mammalian metabolism was criticised.

Clear evidence for a difference between bound products in mammalian and prokaryotic DNA was presented by A. Dipple and coworkers (Frederick Cancer Research Centre, Maryland). Incubation of ^3H -7-12-dimethyl benz[*a*]anthracene (^3H -DMBA) with cultured mouse embryo cells resulted in at least three identifiable DNA reaction products whereas only one of the three was identifiable when two of Ames's *Salmonella* strains were incubated with ^3H -DMBA in the presence of liver microsomes. Because of such deficiencies in prokaryotic test systems, increasing attention is now being paid to eukaryotic and in particular to mammalian systems.

For risk estimates it is important to have precise knowledge of dose-response relationships, and a direct approach to this has been made in several laboratories by the use of cultured human fibroblasts. V. Mayer (Michigan State University) presented data indicating a threshold or no-effect dose for mutation to 8-azaguanine resistance after exposure of human fibroblasts to ultraviolet and some reactive hydrocarbon derivatives. The implications of these observations are of such importance that it is essential they be validated in other laboratories and for other loci, particularly in view of the fact that alterations in methodology can profoundly alter the shape of the dose-response curve for mutation to 8-azaguanine resistance in V79 Chinese hamster cells (S. McMillan, Paterson Laboratories, Manchester). Linear dose-response curves with no threshold have been found for several mutagens tested at both the HGPRT and TK locus in mouse I.5178Y cells (A. Knapp, Department of Radiation Genetics and Chemical Mutagenesis, Leiden) and for 8-methoxypsoralen plus near ultraviolet light tested at the

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HGPRT locus in V79 cells and human diploid fibroblasts (J. Simons, Department of Radiation Genetics and Chemical Mutagenesis, Leiden).

8-methoxypsoralen (8-MOP) plus long wave ultraviolet light is an effective treatment for psoriasis, a debilitating skin disease in man. 8-MOP in the dark is an effective frameshift mutagen in *Escherichia coli* and *S. typhimurium* (B. Bridges, MRC Cell Mutation Unit, University of Sussex). In contrast, base pair substitution mutagenesis occurs after exposure to 8-MOP plus near ultraviolet light. 8-MOP plus near ultraviolet light is mutagenic in both V79 cells and human diploid fibroblasts when assayed at the HGPRT locus (Simons), but no ouabain-resistant mutants could be recovered in V29 cells (C. Arlett, MRC Cell Mutation Unit, Sussex). 8-MOP in the dark does not seem to increase the frequency of sister chromatid exchanges in human lymphocytes *in vitro* although 8-MOP plus near-ultraviolet had a positive effect (A. Brøgger, Norsk Hydro Institute, Oslo). Many assumptions have to be made to estimate risk from such data and essentially negative results for induced chromosome aberrations in lymphocytes of psoriasis patients undergoing treatment have been reported by Brøgger. However, the treatment should be regarded as potentially hazardous although the benefit to individual patients may well offset the overall risk.

Several presentations discussed completely *in vivo* 'short-term' methods for assay of induced somatic mutation in mammals. Mice heterozygous for the *dilute* and *leaden* loci irradiated late in foetal life show patches of abnormal pigment cells when whole mounts of epidermis are made shortly after birth (A. G. Searle, MRC Radiobiology Unit, Harwell). Such changes, the result of recessive mutations at the two coat colour loci, are observable at the cellular level as alterations in the shape of and pigment density in melanocyte precursor cells.

Possible pitfalls in the use of this method were discussed by L. B. Russel (Oak Ridge, Tennessee) who emphasised the need for microscopic scoring, as coat colour spotting on a macroscopic level can be the result of other non-specific effects of mutagen exposure such as decrease in number of precursor cells and/or disturbances in differentiation. With careful attention to such problems, induced mutant frequencies for a number of test compounds roughly paralleled but were significantly higher than those found for germ cell mutations (specific locus test), suggesting that possibly different types of genetic damage are being assayed.

In vivo exposure of animals to mutagens followed by *in vitro* assay is relatively rapid, organ and tissue-specific effects can potentially be monitored, and the approach has the advantage that injected compounds are subjected to full metabolism and detoxification. Using this approach, B. Dean (Shell Research, Sittingbourne) demonstrated increased frequencies of 8-azaguanine and ouabain-resistant clones in cultured cells derived from different tissues of 3-week-old Chinese hamsters previously treated with ethyl methanesulphonate, methyl nitrosourea and diethylnitrosamine.

In vivo exposure and *in vitro* detection is the principle on which monitoring of exposed human populations by scoring chromosome aberrations in lymphocytes is based. For accurate assessment of induced damage it is important that aberrations be scored in the first metaphase after phytohaemagglutinin stimulation. It is now possible to identify first metaphase exactly by culturing lymphocytes in BUdR and identifying second divisions as showing differential staining of chromatids. Using this method, Grossen (Christchurch Hospital, New Zealand) demonstrated that by 48 h, the usual sampling time used, all cultures of lymphocytes examined showed second divisions (range 2–53%). The scoring of such a heterogeneous population could lead to erroneous conclusions with respect to overall sensitivity.

The *in vivo/in vitro* approach was also adopted by R. Albertini (University of Vermont Medical College). This method of mutagen detection, based on autoradiographic measurements of the frequency of phytohaemagglutinin-stimulated lymphocytes which continue to incorporate ³H-thymidine in the presence of 6-thioguanine (6-TG), was validated by the demonstration of a high frequency of such cells in patients suffering from Lesh-Nyhan disease and known to lack HGPRT activity. Significantly higher frequencies of 6-TG resistant lymphocytes were demonstrated in patients receiving cancer chemotherapy. The molecular basis of the 6-TG resistance in this system is at present unknown, but the authors invoke somatic mutation and suggest that the assay could be used in parallel with cytogenetic screening for monitoring genetic damage.

Overall the general feeling was that as yet no single test or panel of tests can accurately predict potential hazards to man, and that 'short-term' tests, particularly in microorganisms, although offering the possibility of rejecting dangerous chemicals at an earlier stage cannot yet completely replace conventional carcinogenic tests with animals.

The concern of industry and the scientific community in this field is indicated by the recent formation of the International Commission for Protection against Environmental Mutagens and Carcinogens (ICPEMC) under the Chairmanship of Professor S. Sobels (Leiden) to act as an advisory body and collate data on an international level. In this way it is hoped that progress can be made in protecting man from his hazardous environment.

Recombination at Nethybridge

from a Correspondent

The fourth bi-annual EMBO Workshop on the Molecular Basis of Genetic Recombination was held at Nethybridge, Scotland, on 20-25 June, 1977. As on previous occasions, a deliberate attempt was made by the organisers to bring together scientists working on a very wide range of recombination systems.

AN essential intermediate in recombination is hybrid or heteroduplex DNA, which may alter its length by branch migration. This involves the breakage and formation of hydrogen bonds between complementary bases at the point where polynucleotide chains exchange pairing partners. For the first time, exact measurements of the rate of branch migration *in vitro* have been made by R. Warner (University of California, Irvine) and C. Radding (Yale University) using ϕ X174 or G4 phage recombination intermediates. The rate of branch migration seems too low to explain the spontaneous formation of long stretches of hybrid DNA *in vivo* and it is therefore likely that the process is driven by enzymes or DNA binding proteins. Electron microscope studies of recombination intermediates in phage PMB-9 by D. Dressler (Harvard University) demonstrate the formation of reciprocal hybrid DNA in molecules held together by a symmetrical strand exchange point. This is dependent on the *recA* function of *Escherichia coli*, but independent of *recB*.

Predictions of the Aviemore model of genetic recombination (proposed by M. Meselson and C. Radding at the second EMBO Recombination Workshop in 1973) were confirmed by experiments in *Ascombolus* reported by J.-L. Rossignol (Université de Paris, Orsay). The model states that recom-

bination is initiated by an asymmetric strand transfer followed by rearrangement, or isomerisation, of polynucleotide chains to give hybrid DNA on two chromatids. The location of hybrid DNA can in part be determined by examining the pattern of gene conversion and post-meiotic segregation (PMS) of individual markers in meiotic octads. Examination of mutants at increasing distances from the point where recombination is initiated, shows that there is an increase in the proportion of aberrant 4:4 octads, which have two PMS events and can only arise from reciprocal hybrid DNA. Furthermore, among a class of mutants that show unequal numbers of 6:2 and 2:6 segregations (gene conversions), the disparity increases with distance from the initiation point of recombination, and Rossignol was able to show that this is expected if hybrid DNA is initially asymmetric, but is later symmetric. In studies on the correction of mismatched bases in hybrid DNA, P. Pukkila (National Institute for Medical Research, Mill Hill) constructed artificial heteroduplex molecules from $\phi 80$ phages containing mutant or wild type tRNA^{Tyr} genes of *E. coli*. Since the tRNA sequences are known, it is possible to construct molecules with known base mismatches. The enzyme DNase I from *Ustilago*, which is implicated in gene conversion, cleaves these molecules at or near the mismatch, but surprisingly, purine/pyrimidine mismatches can be a better substrate than other mismatches. In collaborative studies with M. Meselson (Harvard University), M. Radman (Université Libre, Brussels) reported experiments which may explain the biological function of the mismatch repair system. λ phage heteroduplexes were constructed in which one strand was methylated and the other was not. The *in vivo* correction system appears to remove the mismatched base in the non-methylated strand. The significance of this observation is that newly replicated DNA is non-methylated and therefore the errors in DNA synthesis will be preferentially corrected to wild type. PMS is due to the failure to correct mismatches in hybrid DNA, and R. Holliday (National Institute for Medical Research, Mill Hill) described a new method in *Ustilago* for detecting PMS without tetrad analysis, which may facilitate the search for mutants lacking this particular repair function.

The pairing of chromosomes at meiosis is mediated by an organelle known as the synaptonemal complex (SC). A. Carpenter (University of California, San Diego) described the accumulating evidence in *Drosophila* that electron-dense nodules on the SC are the sites of crossing over. Meiotic

mutants with altered frequency or distribution of cross overs have corresponding changes in nodule formation. In the silkworm *Bombyx*, S. Rassmussen (Carlsberg Laboratory, Copenhagen) reported that nodules are absent from the SC of females where there is no crossing over, but present in males which do recombine chromosomes.

Studies in bacteria of insertion sequences (IS), transposons and phage μ have opened up a new field of *recA* independent recombination events that do not require significant sequence homology. However, the sequence at the ends of a given element (which are often inverted repeats) is essential for the recombinational event promoted by this element. Recent results with IS (J. Besemer, University of Cologne) transposons (N. Kleckner, Harvard University) and phage μ (A. Bukhari, Cold Spring Harbor, New York, and P. Van de Putte, University of Leiden) have shown that in all these systems excision of the element is usually associated with the formation of deletions or substitutions. IS and transposons seem to have preferred regions of integration although there are a number of insertion sites within a given region. It was previously known that integration into host chromosomes is an essential step in the lytic cycle of phage μ , but new isotope experiments indicate that daughter molecules rather than the parental phage genome become integrated. Similarly on induction of a μ lysogen most of the original prophage remains in the chromosome, the daughter molecules being replicated from that template and then reintegrated elsewhere.

The biochemical basis of site-specific *int* mediated recombination has been established by the *in vitro* system of H. Nash described by L. Enquist (both of the National Institutes of Health, Bethesda). A λ circle containing two separated attachment sites, which has been supercoiled by DNA gyrase, is a substrate for reciprocal recombination mediated by the purified *int* protein. Enquist's own results indicate that short regions of hybrid DNA are formed during this type of recombination *in vivo* and that these can migrate from the attachment site if the adjacent region is homologous; in other words, *int* recombination is not absolutely site specific. The basic problem in recombination mediated repair is the mechanism whereby damaged DNA can seek out an undamaged homologous molecule. P. Howard-Flanders (Yale University) now has evidence for a 'transendonuclease' activity in *E. coli*, which is *recA* dependent. In response to psoralen-damaged phage DNA, the enzyme introduces nicks into super-

coiled homologous, but not heterologous DNA *in vivo*, as well as in an *in vitro* system. K. Powell (University of Newcastle-on-Tyne) reported that the *recA* product in *E. coli* is protein X and that the *tif* mutant (which maps in the *recA* gene) produces an X protein with altered electrophoretic mobility in a two dimensional gel system.

Chi mutants in bacteriophage λ are hotspots for recombination, mediated by the bacterial *rec* system. F. Stahl (University of Oregon, Eugene) has now examined λ genomes in which sequences of bacterial DNA have been inserted. Some of these contain naturally occurring *chi* sequences, whereas others do not. He estimates that there is one recombination hotspot for every ten or so bacterial genes. Mutants with properties similar to *chi* are also known in *Neurospora* and *Schizosaccharomyces*, and similar sequences probably occur naturally since it is known that recombination in fungi is distributed unevenly along the chromosome. Moreover, P. Thuriaux (University of Bern, Switzerland) came to the conclusion that recombination may be restricted to structural genes in higher eukaryotes, since neither total map distance of the genome nor the length of individual structural genes varies with DNA content. It is evident that the text-book dogma that recombination is distributed randomly along chromosomes is about to be finally dispelled, both in prokaryotic and eukaryotic organisms. □



A hundred years ago

The Dutch Geographical Society has received a report from the expedition recently sent out to explore Sumatra. One division left Padary in the middle of May for the mountainous centre of the island. They have successfully penetrated into these hitherto unknown regions, and describe them as of surpassing grandeur. The mountain sides are clothed to the very top with a most luxuriant forest growth, almost impenetrable to the sun's rays. The inhabitants consist of a few utterly degraded Malays gathered together in wretched villages. The health of the expedition is excellent.

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review article

Interstellar molecules

B. Zuckerman*

Recently discovered interstellar molecules are a powerful new tool in the arsenal of the astrophysicist. Operating in a completely non-terrestrial environment, interstellar chemistry produces both everyday molecules likely to be found around the house and exotic species never before observed on the Earth. Interpretation of the molecular spectra requires an interdisciplinary approach.

MOST of the mass of our Milky Way Galaxy is contained in the stars. There is also a very cold and tenuous gas between these stars that comprises a small percentage of the total mass of the Milky Way. While not as visually spectacular as the stars and the bright emission nebulae this cold gas has, nonetheless, proved to be an exceptionally powerful tool for the study of such diverse astrophysical problems as cosmology, cosmogony, galactic structure, stellar evolution, star formation and nucleosynthesis. The cold gas, which is composed of atoms, molecules, and 'dust' grains (of uncertain composition), is most effectively studied by means of radio astronomical spectroscopy. Except for a few diatomic molecules, all of the 40-odd known interstellar molecules have been detected only by means of their radio spectra (Table 1). It seems that production, destruction and excitation of these molecules take place in a hostile (by terrestrial standards) environment by means of various processes involving atoms, ions, free radicals, molecular ions and neutral molecules, solid surfaces, cosmic rays and high and low energy radiation. With but one exception the radio spectra of all of the molecules in Table 1 have been detected within the past eight years. Data have accumulated extremely rapidly and the promise for continued rapid growth of the field is bright if large new antennae and interferometers sensitive to millimeter wavelength radiation are constructed. This fluid situation renders a definitive review impossible at this time. I will, here, describe only a few of the more interesting recent developments: those that are basically astronomical as well as those that involve close collaborative efforts among astronomers, physicists and chemists.

Interdisciplinary approach

The most important contribution of the laboratory spectroscopist to the radio astronomer is the measurement and assignment of molecular transition frequencies. Because most of the molecules in Table 1 are stable and have high vapour pressures at room temperatures, it is relatively easy to measure and assign their complete rotational spectra. The astronomer simply uses this type of information to search for and identify interstellar molecules. There are, however, molecules such as free radicals and molecular ions that cannot easily be studied in the laboratory because they are very unstable in laboratory conditions, and others that have very low vapour pressures at room temperatures. Many such molecules have been discovered by accident by

radio astronomers who were searching for other molecules with known spectra. Even in the absence of any laboratory data tentative identification is often possible. Considerations of cosmic abundances of atoms, interstellar chemistry, calculations *ab initio* of molecular structures, isotopic substitution, and hyperfine structure when combined together are often sufficient to identify a molecule with considerable reliability. In this way interstellar HCO^+ , N_2H^+ and HNC were all correctly identified before they were only very recently observed in the laboratory with new experimental techniques¹⁻⁵. The international flavour of the field is highlighted by the nearly simultaneous observations of HNC in laboratories in the USA, West Germany and Australia³⁻⁵. The definite confirmation of the existence of HCO^+ and N_2H^+ in considerable abundances in interstellar molecular clouds show that ion-molecular reactions⁶⁻⁷ must have an important, possibly the dominant, part in interstellar chemistry.

Because of the low kinetic temperatures (10–100 K) and gas densities (10^2 – 10^6 cm⁻³) in most molecular clouds conditions are generally not those of thermodynamic equilibrium. The detailed population distribution in the rotational ladders of interstellar molecules can, in principle, be used to deduce the physical conditions in the clouds (especially the temperatures and densities of both neutral and ionised species). In practice one assumes that, at least microscopically, conditions are in steady state and collisional and radiative rates into and out of the various rotational levels are in balance. Radiative transition probabilities are generally well known. The most abundant constituents of molecular clouds are hydrogen molecules and helium atoms. Neither has a spectrum accessible to radio astronomy, but they play the dominant part in the collisional excitation of the less abundant polar molecules listed in Table 1. Unfortunately, little has been known about the magnitude of and selection rules for collisional transitions under low temperature interstellar conditions.

Recent quantum mechanical calculations⁸⁻¹⁰ of collisional rates between He and HCN, CO and N_2H^+ have removed much of the uncertainty in this area. The propensity rules for the collisional transitions differ from the radiative selection rules and indicate differences between neutral-neutral and neutral-ion collisions as well. This allows the possibility of highly non-equilibrium population distributions, namely inversion (heating) and anti-inversion (cooling), in some rotational transitions. Sometimes this can lead to exotic phenomena such as the high gain SiO, H₂O and OH masers discussed below. Because of the very general competition between ordinary spontaneous radiation, and

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the simple collisions mentioned above, interesting phenomena can occur even over most of the volume of common molecular clouds.

Chemical history of the galaxy

Among the many astrophysical problems that can be investigated with interstellar molecules is the galactic history of the elements¹¹. Stars return a significant percentage of their mass back to the interstellar gas out of which they were born. Red giants and even more evolved stars, such as planetary nebulae and supernovae, are very important participants in this interchanges of mass. The 'cooking' of hydrogen and helium into heavier elements such as carbon, oxygen, and nitrogen deep in the interior of giant stars and the subsequent expulsion of these elements back into interstellar space determines the chemical evolution of our Galaxy. Since it is believed that originally the Universe was composed almost entirely of hydrogen and helium, the formation of planets like the Earth and their living

inhabitants is entirely dependent on this mass exchange between stars and gas.

Recent studies of relative isotopic abundances in various interstellar molecules, such as H_2CO , CO , HCO^+ , HCN and CS^{12-16} , promise to help us unravel the long history of our galaxy. It seems, for example, that there may have been some enrichment of ^{13}C relative to ^{12}C and of ^{17}O relative to ^{16}O in the interstellar medium since the time our Solar System formed some 4.6×10^9 yr ago. With the aid of theoretical calculations on stellar interiors, these observations may tell us about the types of stars that are responsible for the heavy elements found in the interstellar gas. For example, the apparent difference in the $^{15}\text{N}/^{14}\text{N}$ ratio between the central and outer portions of our Galaxy has been interpreted¹⁵ to imply a greater proportion of low mass stars and a lesser proportion of supernovae near the galactic centre.

Unfortunately, a good deal of the interpretation is still beclouded by the possibility that isotopic fractionation effects are important in the formation and/or destruction of interstellar molecules¹⁷. Therefore, it may not be possible

Table 1 Known interstellar molecules as of January 1977

Number of atoms	Molecule	Optical/UV	Radio spectrum	Detected in astronomical objects*
2	H_2 —hydrogen	✓	—	1, 2(?), 3
	OH —hydroxyl radical	✓	Λ -doubling	1, 2, 3, 4
	SiO —silicon monoxide		Rotational	2, 3
	SO —sulphur monoxide		Rotational	3
	SiS —silicon monosulphide		Rotational	2
	NS —nitrogen sulphide		Rotational	3
	CH^+ —methyldiyne ion	✓	—	1
	CH —methyldiyne	✓	Λ -doubling	1, 3
	CN —cyanogen radical	✓	Rotational	1, 2, 3
	CO —carbon monoxide	✓	Rotational	1, 2, 3, 4
	CS —carbon monosulphide		Rotational	1, 2, 3
3	H_2O —water		Rotational	2, 3, 4
	H_2S —hydrogen sulphide		Rotational	3
	SO_2 —sulphur dioxide		Rotational	3
	HCN —hydrogen cyanide		Rotational	1(?), 2, 3
	HNC —hydrogen isocyanide		Rotational	1(?), 3
	OCS —carbonyl sulphide		Rotational	3
	HCO^+ —formyl ion		Rotational	1, 3
	HCO —formyl radical		Rotational	3
	CCH —ethynyl radical		Rotational	1(?), 2, 3
	N_2H^+ —		Rotational	1(?), 3
4	NH_3 —ammonia		Inversion	1, 3
	H_2CO —formaldehyde		K-doubling	1, 3, 4
			Rotational	1, 3
	HNCO —isocyanic acid		Rotational	3
	H_2CS —thioformaldehyde		K-doubling	3
			Rotational	3
5	C_3N —cyanoethynyl radical		Rotational	2
	HC_3N —cyanoacetylene		Rotational	1(?), 2, 3
	HCOOH —formic acid		K-doubling	3
	CH_2NH —methanimine		K-doubling	3
	H_2CCO —ketene		Rotational	3
	NH_2CN —cyanamide		Rotational	3
6	CH_3OH —methyl alcohol		K-doubling	3
			Rotational	3
	CH_3CN —methyl cyanide		Rotational	3
	NH_2CHO —formamide		K-doubling	3
7			Rotational	3
	$\text{CH}_3\text{C}_2\text{H}$ —methyl acetylene		K-doubling	3
	CH_3CHO —acetaldehyde		Rotational	3
			Rotational	3
	NH_2CH_3 —methylamine		Rotational	3
	CH_2CHCN —vinyl cyanide		K-doubling	3
	HC_3N —cyanodiacetylene		Rotational	3
8	HCOOCH_3 —methyl formate		K-doubling	3
9	$(\text{CH}_3)_2\text{O}$ —dimethyl ether		Rotational	3
	$\text{CH}_3\text{CH}_2\text{OH}$ —ethyl alcohol		Rotational	3
	$\text{CH}_3\text{CH}_2\text{CN}$ —ethyl cyanide		Rotational	3

*1, Dark dust cloud; 2, circumstellar envelopes; 3, H II region and/or galactic centre; 4, external galaxies.

to measure directly the $^{13}\text{C}/^{12}\text{C}$ ratio simply by measuring, for example, the abundance ratio $^{13}\text{CO}/^{12}\text{CO}$. Indeed it is strikingly apparent that for at least one important element, hydrogen, isotopic fractionation is very important in molecular clouds. The interstellar D/H ratio has been measured to be $\sim 10^{-5}$ by means of ultraviolet spectrometers on the Copernicus satellite. On the other hand, of the seven deuterated molecules that have been positively or possibly identified in molecular clouds (HD, DCN, DCO^+ , DNC and deuterated water, ammonia, and methylamine), none have yielded abundances, in a ratio to the corresponding non-deuterated molecule, within an order of magnitude of 10^{-5} . HD, for example, also observed in the ultraviolet with the Copernicus satellite¹⁸, is $\sim 10^6$ times less abundant than H_2 , whereas the other six deuterated molecules, all seen by means of their radio spectra, seem typically to be only 10^2 times less abundant than their non-deuterated counterparts.

Despite these obvious fractionation effects, it seems that it may be possible to say something about the D/H ratio as a function of position in our Galaxy. Near the galactic centre DCN seems to be underabundant relative to its abundance in the vicinity of the Sun. This has been interpreted¹⁹ to mean that deuterium itself is underabundant near the galactic centre where most of the observed gas has been processed through stars, in agreement with earlier notions from stellar evolution theory that nuclear burning in stars destroys D rather than produces it. If so, one is tempted to attribute the creation of the interstellar deuterium to the Big Bang explosion. In the simple case of a conventional general relativistic cosmology, a D/H ratio $\gtrsim 10^{-5}$ implies that our expanding Universe is open and will continue to expand forever.

Birth and death of stars

Historically, the stars have not been objects of affection for radio astronomers. Because of their poor angular resolution, radio telescopes are ill-suited to the study of minute objects such as stars, particularly since most stars do not emit much radio energy. Molecular radio astronomy is at a further disadvantage in that bright massive main-sequence stars that are burning hydrogen to helium in their interiors emit so much ultraviolet radiation that all nearby molecules are rapidly destroyed by photo-dissociation. During the past few years, however, it has become evident that the regions in our galaxy where interstellar molecules are observed are usually associated with either the birth or the death of a star or star cluster²⁰.

The most obvious signposts for star formation in our Galaxy are the hot, bright, blue O and B type stars that are 10^4 – 10^5 times as luminous as our sun and which are often surrounded by glowing clouds of ionised gas called H II regions. The Orion Nebula is the most famous H II region in the sky and is visible to the naked eye as the middle 'star' in the sword of Orion, the Hunter. Often associated with these O stars and H II regions are giant molecular clouds, which appear to be fragmented on at least three mass scales: 10^5 , 10^6 , and 10 times the mass of our Sun ($M_\odot$). O and B type stars are often found in associations composed of subgroups separated by 30–60 light years and strung out along the galactic plane²¹. It may be possible to identify strings of giant $10^5 M_\odot$ molecular clouds as the precursors of these OB sub-groups²². Each $10^5 M_\odot$ cloud in turn seems to fragment into $10^3 M_\odot$ pieces separated by distances ~ 3 light years, and which may be precursors of galactic star clusters, such as the Trapezium, which is located at the centre of the Orion Nebula. Near the Orion Nebula, for example, there is a giant molecular cloud that appears to have fragmented into half a dozen or more pieces²³. The fragmentation process may take millions of years.

The $10^3 M_\odot$ pieces fragment further into stars, many of which are seen as infrared continuum sources buried in the

molecular clouds. Often associated with the infrared sources are bright OH (hydroxyl) and water masers, which come from regions comparable with or somewhat larger than the size of our Solar System. Perhaps, if we are both clever and lucky, we shall someday be able to use these masers as a tool for understanding the final stages in the formation of stars and associated planetary systems.

The superficial picture of star formation that we have pieced together above is still riddled with fundamental uncertainties. That is, we still have no clear understanding of motions in molecular clouds and, unlike the main-sequence phase in a star's life, motion is what star formation is all about. The relative importance of magnetic, rotational, gravitational and turbulent motions and forces in molecular clouds is still a subject of hot controversy. A few years ago it was suggested that the broad CO lines observed in molecular clouds could be best explained by free-fall collapse with velocity proportional to radius of large sections of the clouds^{24,25}. But there are problems with such models^{26,27}, including, for example, excessive star formation rates. The recent observation²⁸ of self-reversed CO profiles in some molecular clouds suggest that, at least in these clouds, collapse is not very fast or v is not proportioned to r , or both. Although interpretation of these observations is still ambiguous²⁹, certain asymmetries present in the CO profiles suggest collapse velocities that are comparable with turbulent velocities. But other clouds have been seen with the opposite asymmetries in the CO profiles, thus suggestive of expansion rather than collapse.

If supersonic turbulence is present in molecular clouds, it will die out quickly unless it is regenerated or the clouds are very clumpy. The degree of clumpiness on size scales less than a few tenths of a light year is under investigation^{30,31}. The part, if any, played by magnetic fields is still unclear. Some clever new ideas and/or observations are required if we are to improve our incomplete understanding of star formation.

The death of stars is a somewhat better understood process than their birth. This is because evolved stars, such as red giants, and dying stars, such as planetary nebulae white dwarfs and supernovae, have been studied optically for many years. Except for the white dwarfs, each of these types of star is known to be losing significant mass into interstellar space. Red giants and planetary nebulae are common objects and it is believed that most stars with masses greater than the mass of our Sun pass through both of these stages of evolution. Until very recently, however, it was not clear that anyone had actually seen stars that were evolving between the red giant phase and the planetary nebula phase.

An infrared survey from rockets³² has been followed by ground based infrared^{33–38} and radio molecular^{39–41} observations of some of the very cool stars seen from the rockets. It now seems that the progenitors of planetary nebulae may be observable as very cool, extreme cases of red giant stars. The central stars in these progenitors are enshrouded in a cool circumstellar envelope composed primarily of molecules and dust particles. The presence of the cool dust is deduced from infrared 'excesses', often observed at wavelengths of 3–20 μm . The cool dust absorbs the visible and near infrared light emitted by the underlying star and by hotter dust and reradiates it at longer infrared wavelengths.

The gaseous molecular envelope is best studied by means of radio spectroscopy. Envelopes in red giant stars with more oxygen than carbon ('oxygen-rich') were first detected⁴² eight years ago by means of OH maser radiation that they emit. Later, both water⁴³ and silicon monoxide⁴⁴ masers have been observed from many of these oxygen-rich stars. The apparent sizes of the OH and H_2O emission regions have been measured by trans-continental and inter-continental radio interferometers to be extremely small (of the order of

the size of our Solar System). Because the strongest SiO masers are due to transitions between rotational levels in the first and second excited vibrational states which require energies of thousands of degrees for excitation, there is good reason to suspect that the SiO masers will come from even smaller regions than the OH and H₂O masers. (Because SiO is a stable molecule, it is not unreasonable that it might exist near or even on the stellar surface.)

Because we now know of three different molecules that produce high gain maser radiation in oxygen-rich stars and because the maser levels have different excitation energies, a wide range of conditions in the outer stellar atmospheres and circumstellar envelopes may be investigated with high spatial resolution. With such data in conjunction with the infrared observations we may hope to eventually gain a detailed understanding of mass loss mechanisms and of the physical conditions around evolved oxygen-rich stars⁴⁵. In particular, it is already known that the OH and H₂O maser outputs follow intensity variations in the near infrared flux so that the masers are probably 'pumped' by near and/or far infrared radiation emitted by the star.

The other kind of evolved red giant stars, those with more carbon than oxygen in their outer layers, are rarer than the oxygen-rich stars discussed above. Recent radio observations⁴¹ suggest that many of these carbon-rich stars may be losing mass at a rate comparable with the mass loss rates for planetary nebulae and significantly greater than the rate of mass loss from most oxygen-rich stars⁴⁶. Unfortunately, largely by a quirk of fate, it is not possible to measure the carbon-to-oxygen ratio in planetary nebulae by optical techniques. An interesting problem for future investigation will therefore be to determine if most or only a relatively small fraction of planetaries pass through a carbon star stage.

Comparison of the lifetimes, compositions, distribution, kinematics and masses of planetary nebulae and carbon-rich and oxygen-rich infrared stars should lead to a clearer understanding of the physical mechanism responsible for the ejection of a planetary nebula envelope. It is also possible that the oxygen- and carbon-rich stars form two successive stages in an evolutionary sequence leading to planetary nebulae. It is already known that the distribution and kinematics of the oxygen-rich, variable stars are similar to those of the planetary nebulae. It is therefore conceivable that red giant stars first shed their oxygen-rich outer envelopes at a relatively leisurely pace and thereby reveal a carbon-rich interior region which is then shed more rapidly to form a planetary nebula. Whether this scenario is representative for most or only a few stars awaits further observational tests.

Galactic and extragalactic structure

Because the Milky Way contains an appreciable amount of dust that scatters and absorbs light waves from distant stars, it was not until the advent of spectral line radio astronomy in the 1950s that a complete map of the Milky Way could be constructed. The maps were of the intensity of 21-cm wavelength radio waves emitted by neutral atomic hydrogen located throughout the Galaxy. In conjunction with a simple model for the revolution of mass about the galactic centre, it was possible to plot the distribution of hydrogen atoms in the Galaxy. It was assumed that this map would also represent the distribution of other young constituents in the Galaxy (by 'young' we mean objects such as bright massive stars and their remnants (for example pulsars), which are believed to have formed out of the gas within the past few times 10×10^6 yr). Some astronomers were therefore surprised when the first large scale maps⁴⁷⁻⁴⁹ of the very abundant interstellar carbon monoxide molecules showed a distribution that is very different from the distribution of hydrogen atoms. The

molecular maps indicate that the CO clouds are concentrated near the centre of our pancake-shaped Galaxy, as well as in a ring lying between 4 and 8 kiloparsecs (kpc) from the centre. Very few molecular clouds are located at or beyond the circle of radius 10 kpc that our Solar System traverses as it orbits around the galactic centre. The atomic maps, on the other hand, indicate a fairly uniform density of hydrogen between 4 and 14 kpc from the galactic centre. Therefore, the bulk of the atomic gas lies outside of the solar circle. Since the CO is believed to trace out the distribution of the (unobservable) H₂ molecule, the inescapable conclusion is that atomic (H) and molecular (H₂) clouds in our Galaxy have very different distributions.

The explanation for this difference is not yet certain, but it probably has something to do with the large scale spiral structure of the Galaxy. That is, the 'typical' low density atomic hydrogen cloud, or group of clouds, is not sufficiently massive to become gravitationally unstable and collapse to form denser clouds where H₂ molecules can be formed and protected. Help is needed and such help is probably provided by a spiral density wave that produces shock fronts and compresses the atomic clouds in the inner, but not the outer parts of the Galaxy⁴⁷. Some of the compressed clouds or groups of them become gravitationally unstable and can collapse. (The collapse process might also involve the galactic magnetic fields.) Because current star formation is believed²⁰ to be more closely tied to molecular, rather than atomic clouds, it is not surprising that the best available pictures of the distribution of massive young stars⁵⁰ (indirectly visible at great distances from the sun by the radio waves emitted in the extensive hot plasmas that surround them) and supernova remnants⁵¹ agree much better with the CO maps than with the 21-cm hydrogen maps.

Maps⁵² of the galactic γ -ray (energies ≥ 100 MeV) distribution also show a remarkable resemblance⁵³ to the CO maps. Because the most likely γ -ray production mechanism is interaction of cosmic ray nucleons with interstellar gas nuclei, the γ -ray maps trace out a product of the densities of these two galactic constituents. The data suggest^{53,54} that the cosmic ray intensities are somewhat enhanced in the general vicinity of the molecular clouds and the supernova remnants and that cosmic rays, at least those of energies 1–10 GeV, originate in the Galaxy (as opposed to outside it).

Because of the widespread distribution of CO in the Milky Way, it has been expected that the CO rotational spectrum would also be observed from other galaxies. Recently, however, radio astronomers finally detected extragalactic CO near the centre of one active irregular, four active spiral galaxies⁵⁵, and in the Large Magellanic Cloud⁵⁶, a very nearby irregular galaxy, easily visible with the naked eye from the Southern hemisphere. By 'active' we mean that the galactic central regions emit strong infrared and radio frequency continuum radiation and optical emission lines, as well as showing evidence for non-circular motions and mass ejection. These are also characteristics of the centre of the Milky Way, albeit on a smaller scale. Because the single largest concentration of molecular clouds in our Galaxy is located near its centre, it is not surprising that the nuclei also contain the most impressive concentrations of molecules in the active galaxies. In all of these galaxies, including our own, the molecular clouds and energetic phenomena are probably associated with star formation and death. Gravitational forces tend to enhance the nuclear densities, which result in massive molecular clouds and relatively rapid rates of formation of stars which excite the interior regions and might lead to violent (explosive) events such as supernovae. With somewhat more sensitive receivers, millimetre wavelength interferometers, and larger tele-

scopes it should be possible to greatly expand our knowledge of the distribution of extragalactic molecules. Higher angular resolution, for example, would enable radio astronomers to map the (spiral) structure of other galaxies and to compare the molecular distribution with the distribution of dust, atomic gas, emission nebulae, and synchrotron radiation. Better sensitivity will allow study of the less abundant isotopes of CO and other molecules which in turn will help us to understand the chemical history of these galaxies.

Summary

The study of interstellar molecules during the past decade has been remarkably productive. Future prospects look equally bright. New large telescopes and interferometers sensitive to millimetre wavelength radiation will permit substantially more detailed study of molecules in regions of stellar birth and death in the Milky Way and external galaxies. New instrumental techniques have already pushed the short wavelength frontier to sub-millimetre wavelengths (the observation⁵⁷ of the J=3→2 transition of CO on the 200-inch Hale telescope). Portions of the millimetre and sub-millimetre spectrum, not accessible from the ground because of atmospheric absorption, will be observable from aircraft, balloons and satellites. Initial steps in this direction have been taken⁵⁸ with a 183 GHz radiometer flown on the G. P. Kuiper Airborne Observatory.

Perhaps the most fertile, as yet largely unexplored, area of the spectrum is the infrared, where high resolution spectroscopy is still in its infancy. H₂ rotation-vibration transitions near 2-μm wavelengths have recently been detected⁵⁹ for the first time in interstellar space towards the Orion Nebula and a planetary nebula.

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articles

β-Sheet topology and the relatedness of proteins

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The topological connectivities of β-pleated sheets in the known protein structures are systematically surveyed for regularly occurring features. The many empirical restrictions found and the distribution of actually occurring topologies among the hypothetically possible ones suggest that striking topological similarities between different proteins can readily happen by chance.

β-PLEATED sheets are major features in about 80% of the globular proteins of known three-dimensional structure, and β sheets inherently require the close interaction of regions separated in the amino acid sequence. An understanding of β sheets is therefore central to an understanding of the organisation and folding of proteins, and will probably be essential in any successful method of predicting three-dimensional structure from amino acid sequence. A number of particular regularities have been noted in β structure¹⁻⁴, but there has been no systematic search for such features.

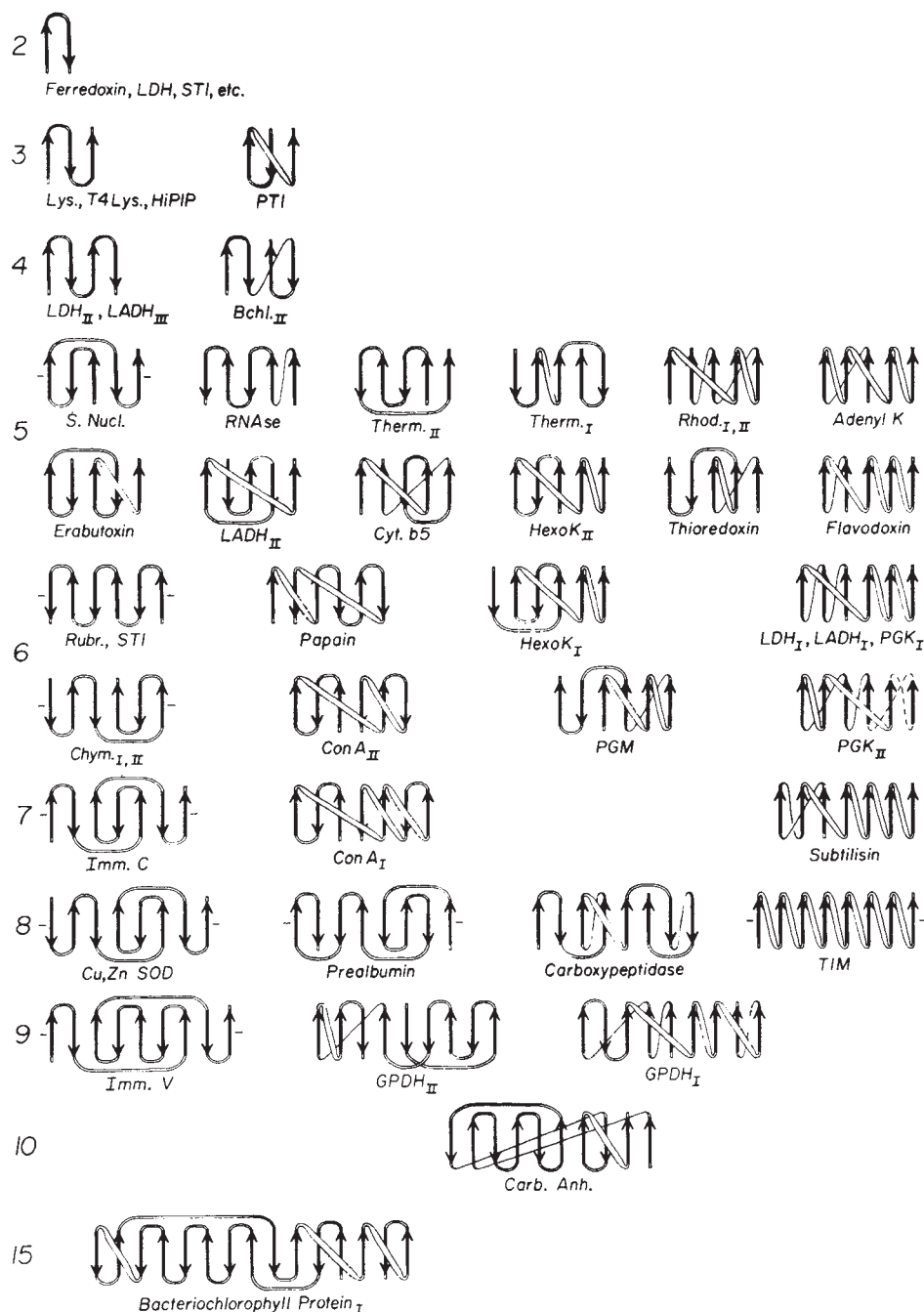


Fig. 1 Schematic diagrams for all the topologically-distinct β -pleated sheets found in proteins of known three-dimensional structure. Arrows indicate the strands of the β sheet, considered to be in the plane of the paper; crossover connections shown wide are above the sheet and thin ones are below it. Only topological connectivity is represented, with no attempt to indicate the length or conformation of the connections. The β sheets are sorted from top to bottom by number of strands, and from left to right according to their percentage of antiparallel as against parallel β structure. In cases of proteins with more than one β sheet, roman numeral subscripts are added. Those β sheets which can be considered to form cylindrical barrels are flanked by hyphens and are shown as viewed from the outside. Abbreviations and literature references for the 33 proteins included (in decreasing order of β -sheet size) are as follows: bacteriochlorophyll protein (Bchl.)⁸, carbonic anhydrase C (Carb. anh.)⁹, glyceraldehyde phosphate dehydrogenase (GPDH)¹⁰, immunoglobulin variable (Imm. V) and immunoglobulin constant (Imm. C) domains¹¹, Cu,Zn superoxide dismutase (Cu,Zn SOD)¹², prealbumin¹³, carboxypeptidase A¹⁴, triose phosphate isomerase (TIM)¹⁵, concanavalin A (Con A)¹⁶, subtilisin¹⁷, chymotrypsin (Chym.)¹⁸, phosphoglycerate mutase (PGM)¹⁹, phosphoglycerate kinase (PGK)²⁰, rubredoxin (Rubr.)²¹, soybean trypsin inhibitor (STI)²², papain²³, hexokinase (HexoK)²⁴, lactate dehydrogenase (LDH)²⁵, liver alcohol dehydrogenase (LADH)²⁶, erabutoxin²⁷, cytochrome b5 (Cyt. b5)²⁸, thioredoxin²⁹, flavodoxin³⁰, staphylococcal nuclease (S. nucl.)³¹, pancreatic ribonuclease (RNase)³², thermolysin (Therm.)³³, rhodanese or thiosulphate sulphurtransferase (Rhod.)³⁴, adenyl kinase (Adenyl K)³⁵, egg white lysozyme (Lys.)³⁶, T4 phage lysozyme (T4 Lys.)³⁷, high-potential iron protein (HiPIP)³⁸, pancreatic trypsin inhibitor (PTI)³⁹, ferredoxin⁴⁰.

Similarity of the topological connectivity of β -sheet strands has been central to consideration of the possible evolutionary relationship between distantly similar proteins⁵⁻⁷, but it has been very difficult to evaluate the significance of such topological similarities. Here I introduce both a graphical and a notational convention for representing the topological connectivity of β sheets, and use this highly

simplified representation to systematically survey the known β sheets for regular patterns. Possible implications for the folding and for the evolutionary relatedness of proteins are pointed out.

The possible backbone connections between β strands fall into two general categories: 'hairpin' connections in which the backbone chain re-enters the same end of the β

sheet it left, and 'crossover' connections in which the chain loops around to re-enter the sheet on the opposite end (illustrated in Fig. 1 of ref. 2). Starting from the N-terminal strand, each connection is named by how many strands it moves over in the sheet and in which direction, with an 'x' added for crossover connections. Thus a '+1' is a hairpin and a '+1x' is a crossover connection between nearest-neighbour strands; a '+2' is a hairpin and a '+2x' is a crossover connection that skips past one intervening strand in the sheet, and so on. As an example, the five-stranded β sheet of thioredoxin (see Fig. 1) can be described as either $-2x, +1x, -2, -1$ or as $+2x, -1x, +2, +1$ (absolute value of the signs is not meaningful, since the diagram could be turned upside down).

Figure 1 shows simplified topological diagrams for the 37 topologically distinct β -sheet structures found in proteins of known three-dimensional structure. Assignment of these β -sheet topologies was made from examination of stereo drawings, hydrogen-bonding diagrams, and published descriptions of the structures. In some cases a decision must be made about inclusion or omission of a particular extended strand: very short strands which turn abruptly away after making one hydrogen bond were generally omitted if they were at an edge of the sheet, while long extended strands which stay close to a neighbouring strand are generally included even if they are not known to form hydrogen bonds (for example, a fifth parallel strand in rhodanese). Also, each continuous stretch of extended chain is treated as a single β strand; this is the usual convention but differs, for example, from the usage in ref. 4. All of these β sheets have the usual twist¹, although that is not shown in these schematic diagrams. All consideration of length and conformation in the connecting backbone chains is omitted, in order to concentrate on the purely topological features. Figure 1 thus summarises the empirical data base from which all of the following conclusions are drawn.

Handedness of crossover connections

All crossover connections are right-handed (except for one, in subtilisin). That is, in Fig. 1, crossover connections above the β sheet all slant down to the right, and crossover connections below the sheet all slant down to the left. This handedness phenomenon is discussed in detail elsewhere².

Parallel β structure only in large, protected sheets

No parallel β structure occurs in sheets of less than five total strands. This suggests that considerable cooperativity is necessary to stabilise parallel β structure, whereas antiparallel β structure often occurs as a twisted ribbon of just two strands.

Parallel β sheets and the parallel portions of mixed sheets are always thoroughly buried, with other main chain (often α helices) covering them on both sides. Antiparallel sheets, on the other hand, typically have one side exposed to solvent with only the rather sparse protection of the side chains. This pattern also suggests a lower stability for parallel β structure, since apparently it cannot tolerate solvent access to its hydrogen bonds. The necessity for considerable protein structure on both sides of a parallel β sheet (as well as the need for loops to make the crossover connections) means that parallel sheets occur only in relatively large protein domains. For domains built around a parallel β sheet the average size is 179 residues with a minimum of about 135, whereas for domains built around

a large antiparallel sheet the average size is 128 residues and the minimum is 54.

For right-handed crossover connections to shield both sides of a non-cylindrical parallel β sheet, there must be at least one change of direction (change of sign in the written convention). Apparently these direction changes are somewhat unfavourable for parallel sheets, since none of the nine pure parallel β sheets change direction more than once, although for antiparallel and mixed sheets of similar sizes 11 out of 20 examples change direction two or three times.

	$\uparrow\downarrow$	Mixed	$\uparrow\uparrow$	Total
$\uparrow\downarrow \pm 1$	57	48		105
$\uparrow\uparrow \pm 1x$		12	32	44
$\uparrow\downarrow \pm 2$		9		9
$\uparrow\uparrow \pm 2x$	7	8	7	22
$\uparrow\downarrow \pm 3$	8	4		12
$\uparrow\uparrow \pm 3x$		7	5	12
$\uparrow\downarrow \pm 4$		2		2
$\uparrow\uparrow \pm 4x$	4			4
$\uparrow\downarrow \pm 5$		3		3
$\uparrow\uparrow \pm 7$		1		1
$\uparrow\downarrow \pm 7x$		1		1
$\uparrow\uparrow \pm 8x$		1		1

Fig. 2 Occurrence frequencies of each connection type in the known β sheets. The number of occurrences in pure antiparallel, in mixed, and in pure parallel β sheets are tabulated separately, and are totalled for all β sheets of 5 or more strands.

Connection type frequencies

Figure 2 gives the number of occurrences of each connection type. Of the connections that occur, 70% are either ± 1 or $\pm 1x$. This fact alone is sufficient to make some β -sheet topologies much more favourable than others, as has previously been pointed out⁶.

Asymmetries of chain direction

For almost all local (2 or 3 strand) topological features, occurrence frequencies seem to be indifferent to backbone chain direction. One exception is that the N-terminal strand is somewhat more likely than the C-terminal strand to be in the middle of the β sheet: 15 N-terminal strands are at an edge, 4 are in by one strand, and 14 are in by more than one strand; while 22 C-terminal strands are at an edge, 7 are in by one strand, and only 4 are in by more than one strand. This supports the view that there is a tendency for the earliest folding nucleation sites to be near the beginning of the chain.

In general, reversal of chain direction produces a topologically distinct structure (except in occasional symmetrical cases, such as chymotrypsin and the immunoglobulin domains); however, since occurrence frequencies of all the local patterns except the buried N termini are indifferent to chain reversal, the following discussion assumes that a given topology and its reversed version have the same probability of occurrence.



Fig. 3 Comparison of geometric motifs common on Greek and American Indian weaving and pottery with the backbone folding patterns found for cylindrical β -sheet structures in globular proteins. *a*, Indian polychrome cane basket, Louisiana. *b*, Polypeptide backbone of rubredoxin. *c*, Red-figured Greek amphora showing Cassandra and Ajax (about 450 BC). *d*, Polypeptide backbone of prealbumin. *e*, Early Anasazi Indian redware pitcher, New Mexico. *f*, Polypeptide backbone of triose phosphate isomerase. Photographs (*a*) and (*e*) courtesy of the Museum of the American Indian, Heye Foundation; photograph (*c*) courtesy of the Metropolitan Museum of Art, Fletcher Fund, 1956.

Simplicity of commonest topological patterns

Among locally interconnected 4-strand regions, the only topologies that occur more than 3 times are: $+1, +1, +1$ 16 times; $+3, -1, -1$ 11 times; $+1x, +1x, +1x$ 6 times; and $+1x, +2x, -1x$ 5 times. These frequencies are about what would be expected from the occurrence frequencies of the individual connection types making them up (see Fig. 2), except that $+3, -1, -1$ occurs more often than expected. Perhaps one reason why that pattern is especially favoured is that besides folding up one strand at a time it can also fold by forming a long 2-stranded ribbon which then bends in the middle.

The distinctive topological patterns which are seen in the β -barrel structures are the simple up-and-down 'meander' pattern of $+1, +1, +1$ (as in rubredoxin and soybean trypsin inhibitor); the 'Greek key' pattern of $+3, -1, -1, +3$ or just $+3, -1, -1$ (as in staphylococcal nuclease, chymotrypsin, immunoglobulins, superoxide dismutase and prealbumin); and the strict alternation of parallel β with helix in the 'lightning' pattern of

$+1x, +1x, +1x$ (as in triose phosphate isomerase). As shown in Fig. 3, each of these patterns corresponds to a common geometric motif used to decorate weaving and pottery. Besides providing a useful mnemonic for the β -barrel structures, this comparison emphasises the fact that, like good pottery decoration, the topological patterns found in β structure are not haphazard collections of lines but are subject to constraints of simplicity and good design.

For parallel β barrels, since crossover connections must be right-handed and cannot go down the centre of the barrel, a rigorous repetition of $+1x, +1x, +1x$ in the lightning pattern is the only likely topology. Therefore it is to be expected that any parallel β barrels found in the future will very closely resemble triose phosphate isomerase, whether they are related to it or not.

The Greek key topology of $+3, -1, -1, +3$ is symmetrical to chain reversal, but when it occurs on a barrel it has two mirror-image forms, depending on whether the swirl is clockwise or counterclockwise as viewed from the outside of the barrel (see Fig. 3 insert and cover). For all five proteins with Greek key β barrels, the swirl is counterclockwise. This could simply be a strong coincidence; however, it seems likely that the counterclockwise swirl either folds more easily or is a stable structure (the two forms are not exact mirror images, because the β strands always have a right-handed twist and the amino acids are asymmetrical).

Preference for pure over mixed sheets

The ratio of the number of pure parallel or pure antiparallel β sheets to the number of mixed sheets is very much greater than would be expected from a random distribution. In our sample there are 124 antiparallel and 73 parallel strand pairs in the sheets of at least 5 strands. If the parallel and antiparallel probabilities are considered approximately equal and if they were independent from one pair to the next across the β sheet, then there should be approximately 2 pure sheets (one parallel and one antiparallel) for every 14 mixed sheets in 5-stranded cases, and 2 pure for every 30 mixed in 6-stranded cases. Since Fig. 1 contains 12 5-stranded and 8 6-stranded sheets, the expectation would be 2 pure and 18 mixed sheets if the probabilities were independent; in fact there are 12 pure and only 8 mixed sheets out of the 20.

It is easy to understand why the parallel and antiparallel probabilities are not independent from one strand pair to the next: since the ideal conformations for the two types of β structure are slightly different, a strand which has antiparallel hydrogen-bonding on one side is already set up for antiparallel hydrogen bonds on the other side. Indeed, it turns out that 138 strands have the same type of bonding on both sides, while only 33 have opposite bonding types on the two sides.

One interesting result of this strong preference for pure rather than mixed β sheets is that although the current set of known protein structures is beginning to provide a statistically meaningful sample of the pure β sheets (18 with >4 strands), the 14 mixed sheets are actually 15 times sparser sampling out of the possible cases, and therefore very little can yet be said about them.

Absence of knots

There are no knotted topologies within the known β sheets—that is, no backbone β -sheet configurations which, if considered as a rope grasped at N and C termini and pulled out straight, would end up with a knot in the rope. (However, the backbone of carbonic anhydrase C contains a knot involving the first 30 residues (not part of the β structure) and the last 20 residues (which do include a β strand). (Presumably a knot is not impossible

if the piece to be tucked through the loop is very close to a chain end.) It has often been pointed out (for example ref. 41) that it would be exceedingly difficult for a protein to fold up into a knotted configuration. This is not a trivial restriction, since a relatively large number of the hypothetically possible topologies are knotted, especially for parallel sheets. The simplest such case is $+2x, -3x, +2x$, —the one knotted topology out of 12 (if left-handed crossover connections are excluded) for a 4-stranded pure parallel sheet. Of the possible topologies, 25% are knotted for a 5-stranded pure parallel sheet, and 45% of the topologies are knotted for a 6-stranded pure parallel sheet.

Adequacy of a simple nomenclature

One set of restrictive conditions can be summarised by saying that it is almost always possible to describe a real β -sheet topology by a list of connection types. Many of theoretically possible arrangements which would violate this restriction simply do not occur: for instance, two long β strands hydrogen bonded to each other as nearest neighbours at one end but hydrogen-bonded to an intervening β strand at the other end.

Ambiguous chain crossings are also extremely rare in real structures (that is, connections which would be topo-

suggested before^{2,42-45}. Assuming that the α helices and β strands are already present at least statistically, let us say that each succeeding step in any possible folding pathway for a β sheet must consist of either (1) forming a ± 1 or $\pm 1x$ connection between two β strands adjacent in sequence, or (2) taking either a β strand or a prefolded unit and laying it down next to a prefolded part of the sheet with which it is also contiguous in sequence. Many hypothetical topologies which do not occur are unfoldable by this definition, including all the knotted ones. But the occurring β sheet topologies typically have a large number of such possible folding pathways (the notable exceptions are subtilisin, with no such pathways because the left-handed $+1x$ lies outside of the $-2x$, and cytochrome b5, with only one). Especially for cases with 10 or 20 potential folding pathways it seems unlikely that one is so much more favourable that it effectively dominates all the others. Instead, it may be that one reason that these structures are favourable is because they can fold correctly by many alternative pathways and therefore tend to fold rapidly and reliably. The idea that some broad features of protein structure are determined by essentially kinetic factors during folding (such as the dominance of near-neighbour strand interactions) is not incompatible with the requirement that the stable native conformation of a protein should be in the global free energy minimum. During its evolution, once a protein finds a reasonably stable, kinetically accessible, minimum energy conformation, it is then subject to natural selection for stability. Selection will dig that particular local energy minimum as deep as possible by adjusting the amino acid sequence to approach optimal fit for the native conformation, and at the same time probably will raise all the other local energy minima whose stability is not being selected for. The result is very likely to be a genuine global energy minimum, in spite of the influence of kinetic requirements.

	$+1, +1$	41		$+1x, +1x$	15
	$+1, +1x$	3		$+1x, +2$	2
	$+1, +2$	5		$+1x, -2$	3
	$+1, -2$	3		$+1x, +2x$	10
	$+1, +2x$	4		$+1x, -2x$	10
	$+1, -2x$	7		$+1x, +3$	1
	$+1, +3$	10		$+1x, +3x$	8
	$+1, -3$	13		$+1x, -3x$	4
	$+1, +3x$	3		$+2x, +2$	2
	$+1, -3x$	4		$+3x, -2$	1
	$+1, -4$	2		$+4x, -3$	1
	$+1, -4x$	3			

Fig. 4 Occurrence frequencies for consecutive (in the amino acid sequence) pairs of connection types in β sheets of five or more strands. A connection pair and its reversal (for instance, $+1x, -2x$, and $-2x, +1x$) are tabulated together. Connection pairs that span more than five strands are omitted.

logically different depending on which of two connection chains was on top). There is just one such case involving hairpin connections in GPDH_{II} (see Fig. 1), and just one such case involving crossover connections, in subtilisin (see Fig. 1). Often one version of a hypothetical ambiguous pair is knotted; the two versions are not counted as separate topologies in this paper.

Preference for topologies with many folding pathways

It is possible to assume a hypothetical set of rules for allowable steps of protein folding for β sheets, more or less in agreement with ideas which have often been

$+1x, +1x, +1x, +1x$	50		TIM
$+1x, +1x, +2x, -1x$	44		(LDH, LADH) Flavodoxin (PGK _I)
$+1x, +2x, +1x, -2x$	30		Adenyl K
$+1x, +2x, -1x, +2x$	30		
$+3x, +1x, -2x, -1x$	24		Rhodanese PGK _{II}
$+2x, +1x, +1x, -3x$	18		
$+1x, +3x, -1x, -1x$	14		LDH, LADH PGK _I
$-1x, +3x, +1x, -2x$	10		

Fig. 5 The most probable topologies for 5-stranded pure parallel β sheets. The first column lists the sequence of connection types. Numbers in the second column are normalised pseudo-probabilities obtained by multiplying occurrence frequencies of the three pairs of connection pairs as described in the text. The diagrams in the last column are obtained by reversal of the backbone chain direction. Next to each diagram are listed all proteins which contain that topology as a contiguous unit (both in sequence and within the β sheet), the protein is in parentheses if a single strand at one end of either the sequence or the sheet was ignored to make the 5 strands contiguous. See Fig. 1 legend for abbreviations.

Probability ordering for β topologies and implications for evolutionary relatedness

Figure 4 gives the occurrence frequencies for various consecutive pairs of connection types. These pair frequencies can be used to generate pseudoprobabilities which give a relative ordering among possible β -sheet topologies.

As an example, consider the pure parallel and the pure antiparallel cases, for 5-stranded β sheets. With left-handed crossover connections eliminated (see above), parallel and antiparallel cases each have $n!/2 = 60$ possible topologies, consisting of 32 basic patterns and 28 others obtained by reversal of chain direction (4 of the topologies are symmetrical to chain reversal). Each 5-stranded topology contains 4 connections and 3 connection pairs. Its pseudoprobability is defined as the number obtained by multiplying together the occurrence frequencies of its three connection pairs (from Fig. 4, using a frequency of 1 if the pair did not occur in the data base), dividing by 2 for the symmetrical cases, and then dividing by a factor of 34 which normalises the +1,+1,+1,+1 case to 1,000.

Figures 5 and 6 show all pure parallel and all pure antiparallel 5-stranded topologies that have pseudoprobabilities greater than 8. The data sample is large enough (155 connection pairs) so that the pseudoprobabilities respond primarily to the overall connection pair frequencies and are only slightly affected by whether or not the exact 5-stranded topology in question occurs in the data base. The pseudoprobabilities could be further improved by inclusion of factors disfavouring knots, ambiguous chain crossings and buried C termini (see above). Also, for purposes that did not need to consider topologies embedded within larger sheets, it would be useful to include the probability of each connection type being the first or the last. But, the single simple criterion used in Figs 5 and 6 succeeds in including all occurring 5-stranded topologies on a rather short list, and the occurring topologies are a fairly dense sample of these 'most favourable' cases.

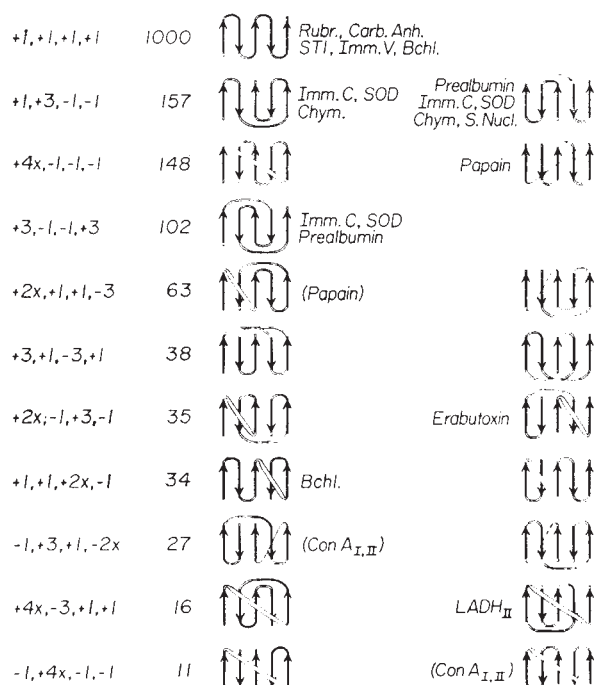


Fig. 6 The most probable topologies for 5-stranded pure anti-parallel β sheets. All pure five-stranded topologies which occur as contiguous units in one of the known β sheets are included on the lists of Figs 5 and 6, except for the case of subtilisin, which would match the adenyl kinase entry if it did not have a left-handed crossover connection.

From the overall distribution of examples seen in Figs 5 and 6, it can be concluded that many of the protein pairs which have identical or closely similar β -sheet topologies merely represent coincidentally close choices among the rather few highly probable topologies. There must be examples among such proteins which resemble each other because of a true evolutionary relationship; however, topological similarity of their β structures provides only a very weak argument for such evolutionary relationship, although it is probably a necessary condition. It is suggestive that fully 75% of the pure β sheets are topologically similar to some other one, whereas among the mixed β sheets, for which the current sampling is 15 times sparser (see above), the only topologically similar pair are the two halves of hexokinase. At one extreme, evolutionary relationships almost certainly underlie the similarity of the nucleotide-binding domains of LDH and LADH, and the internal duplications of structure in rhodanese and in the immunoglobulins. At the other extreme, there are almost certainly not evolutionary relationships underlying the topological similarity of the two interdigitated β sheets in con A or underlying the topological similarity of proteins whose overall structural organisation is as different as, for instance, staphylococcal nuclease and Cu,Zn superoxide dismutase. The many cases intermediate between these extremes must each be decided on the basis of other evidence besides topology, but it seems likely from overall statistics that the bulk of them are coincidences produced by the fact that the number of highly favourable chain folding patterns is actually quite limited.

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Nerve growth factor-induced increase in electrical excitability and acetylcholine sensitivity of a rat pheochromocytoma cell line

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A cloned line of rat pheochromocytoma cells (PC12) which responds to nerve growth factor (NGF) by cessation of division and extension of neurone-like processes, is shown to develop electrical excitability and greatly increased acetylcholine sensitivity in the presence of NGF. This implies that NGF influences the physiological differentiation of certain neural crest derivatives.

LITTLE is known about the mechanisms which regulate the differentiation of neuroblasts into mature neurones. Cell cultures of normal or neoplastic origin have become increasingly useful model systems with which to study this otherwise difficult problem¹. Recently a clonal cell line of rat pheochromocytoma cells (named PC12) was established which undergoes neurone-like morphologic differentiation in the presence of nerve growth factor (NGF) (ref. 2). NGF is a well characterised protein which stimulates neurite outgrowth from sympathetic neurones and which is thought to have an important role in their normal growth and development³. After several days in the presence of 10^{-10} M NGF, PC12 cells cease to divide and extend long neurite-like processes. In common with sympathetic neurones, PC12 cells (both NGF-treated and -untreated) also synthesise, store, take up, and release catecholamines^{2,4,5}. Such findings suggest that in the presence of NGF, PC12 pheochromocytoma cells differentiate into sympathetic-like neurones.

Among the most characteristic properties of differentiated nerve cells are their electrical excitability and sensitivity to neurotransmitters. In this study, we have therefore chosen to establish whether PC12 cells would generate all-or-nothing action potentials, if they would respond to acetylcholine (ACh) and if these properties were affected by NGF.

PC12 cells were grown in 35-mm collagen-coated Falcon plastic tissue culture dishes as previously described². 2.5×10^5 NGF (ref. 6) at 50 ng ml^{-1} was added to some cultures. The cells grown without NGF remained relatively spherical (between 6–14 μm in diameter) and did not extend processes. Cells grown in the presence of NGF for longer than 1–2 weeks were, on the average, slightly larger (10–15 μm in diameter) and most cells had one or more long processes. Physiological experiments were performed at 35–37 °C in medium containing 6 mM glucose, 145 mM Na, 3 mM K, 1.8 mM Ca, 1.0 mM Mg, 153 mM Cl buffered by 2.5 mM phosphate or 2.5 mM Tris to pH 7.4. The cultures were placed on the stage of an inverted phase contrast microscope and the cells were impaled under direct vision with microelectrodes containing 3.5 M potassium acetate ($R = 40\text{--}100 \text{ M}\Omega$) (ref. 7). The cells were stimulated with intracellular depolarising pulses of 50-ms duration passed through the recording electrode using a modified Wheatstone bridge. In many cases, in order to increase activation of spike generating mechanisms, the cells were hyperpolarised by passing

negative background current through the microelectrode before depolarising pulses were applied.

Since PC12 cells are small and therefore subject to injury with the microelectrode, several criteria were used to control for this parameter when comparing spike generative capacity and ACh sensitivity of cells cultured in different conditions. First, recordings were included in our results only if the cell maintained a resting potential of -20 mV or greater. This excluded severely damaged cells. Second, the mean resting potential of each group of cells was determined. While such measurements do not reflect the absolute resting potentials of the unimpaled cells, they do provide a means of comparing the extent of cell damage. Finally, in many cases, comparisons were made with cultures studied on the same day and with the same set of microelectrodes.

Electrical excitability

In more than one hundred impaled PC12 cells with resting potentials greater than -20 mV , from eight different cultures, no cells showed action potentials (Fig. 1a and Table 1). This included 58 cells with membrane potentials (V_m) greater than -30 mV and 19 cells with V_m greater than -40 mV . In all cases, the cells were hyperpolarised to between -70 and -100 mV in order to maximally activate any possible spike-generating mechanism present. In two cells, after background hyperpolarisation, depolarising pulses produced small regenerative responses ($<10 \text{ mV}$) which could just be distinguished from the delayed rectification which was often present.

In some experiments, more calcium (5.4–10.8 mM) was added to the medium to act as a membrane stabiliser and to enhance any Ca-dependent regenerative responses. Action potentials were not detected in 18 impaled cells (including 9 cells with $V_m \geq -30 \text{ mV}$ and 5 with $V_m \geq -40 \text{ mV}$) in these conditions.

As noted above, delayed rectification responses were frequently observed (Fig. 1a) indicating that the mechanism controlling the delayed increase in K conductance of action potentials is present in these cells. Previous experiments have suggested that PC12 cells not treated with NGF also possess at

Table 1 Electrical excitability of PC12 cells grown in different conditions

Growth condition	Cell division	Process	Action potentials*	Mean V_m
NGF-untreated	+	—	0/111	31 ± 1
NGF-treated (> 2 weeks)	—	++	29/42	33 ± 2
Ara-C (0.25–3 μM)	—	—	11/91	38 ± 1
BUdR (3 μM)	—	—	0/15	35 ± 2
Ara-C+NGF (> 2 weeks)	—	++	49/65	44 ± 1

*Numbers indicate cells with action potentials per total cells sampled with V_m equal to or larger than -20 mV .

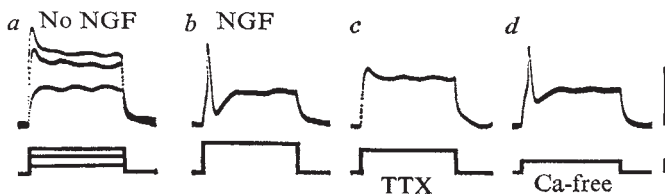


Fig. 1 Electrophysiological responses of PC12 cells. *a*, PC12 cell not treated with NGF. Three superimposed intracellular depolarising pulses (voltage trace above; applied current below) do not evoke a regenerative response. Delayed rectification is seen in the two larger responses ($V_m \approx -62$ mV*). *b-d*, PC12 cells treated with NGF for more than 2 weeks. *b*, Depolarising pulse evokes action potential in PC12 cell bathed in control medium ($V_m \approx -58$ mV*). *c*, Depolarising pulse fails to evoke regenerative response in another PC12 cell in presence of 3×10^{-7} M TTX. ($V_m \approx -54$ mV*). *d*, PC12 cell bathed in Ca-free medium; action potential similar to that seen in *b*. ($V_m \approx -70$ mV*). Calibration is 50 mV, 1 nA and depolarising pulse is 50 ms. (*, Indicates approximate V_m with background hyperpolarisation).

least some Na action potential ionophores, since veratridine, an alkaloid which specifically activates such sites⁸, stimulates release of endogenous catecholamine stores⁴. The inexcitability of untreated PC12 cells could derive from too low a density of such ionophores or from the absence of another component of the action potential generating mechanism or for some other reason.

In PC12 cultures maintained in the presence of NGF for more than 2 weeks, most impaled cells demonstrated all-or-nothing action potentials when depolarised by passing current through the microelectrode (Fig. 1*b* and Table 1). Most often the cells required some background hyperpolarisation to between -50 and -100 mV before the depolarising pulse could elicit an action potential. Resting membrane potentials varied between -20 and -70 mV. In two experiments combined, 22 of 25 consecutively impaled cells showed active responses. In other experiments, presumably because of injury, active responses were found in only 50–80% of penetrated cells. The action potential morphology exhibited by these cells was varied but no cells showed prolonged plateaus, as may be seen in some murine neuroblastoma cells⁹. It could not be determined how much of this variability was attributable to differences in the population or was a function of impalement injury.

Most of the cells in the NGF-treated cultures had long (>100 μ m) processes and thus it was possible that action potential generation was directly related to neurite outgrowth. In order to test this, those few cells in NGF-treated cultures which lacked visible processes were impaled. Such cells were still capable of generating action potentials. In another experiment, cells were grown in NGF for 3 weeks and then detached from the culture dish and subcultured into new media with NGF. As a result of this treatment, the cells lost their processes, mainly by mechanical shearing. Within 24 h, the process-less cells had settled on the dish and only a few began to extend short processes. Despite the lack of extensive process formation, at least 60% of the cells were electrically excitable. Both of these experiments indicate that process outgrowth, *per se*, is not responsible or necessary for the development of the action potential mechanism.

The action potentials in the PC12 cells were readily abolished by tetrodotoxin (3×10^{-7} M), an agent known to block Na-action potentials (Fig. 1*c*). In one experiment, action potentials were elicited from 11 of 12 impaled cells in control medium (mean $V_m = -27$ mV), none of 15 cells in TTX-containing medium (mean $V_m = -30$ mV), and 7 of 14 cells in control medium after washout of TTX (mean $V_m = -27$ mV). There were no residual regenerative potentials seen in TTX, as are seen in murine C1300 neuroblastoma cells⁹. Action potentials could also be recorded in PC12 cells in Ca-free solution (Fig. 1*d*). The TTX and Ca-free solution data indicate that the PC12

action potential is generated predominantly by a Na mechanism. Similar results have been found for action potentials of sympathetic neurones¹⁰. We did not attempt to elicit action potentials in TTX and high Ca, so we cannot exclude a small Ca component to the action potential. Our data indicate that any such component would not contribute a significant amount to the action potential at physiological ionic concentrations.

When NGF is added to PC12 cultures, cell division stops within 4–5 d and the cells begin to put out processes after this time. By approximately 16–18 d most of the cells have processes. We have recorded from cells in sister PC12 cultures after varying days in NGF. Figure 2 shows the percentage of electrically excitable cells in cultures treated with NGF for up to 3 weeks. Because of injury to many cells, each point may be an underestimate, but there is no indication on the basis of the mean resting potentials that NGF protected the cells from impalement injury. The time course for the development of electrical excitability roughly parallels that of the outgrowth of processes².

Response to iontophoretically applied acetylcholine

Acetylcholine (ACh) is the depolarising neurotransmitter for both adrenal chromaffin cells and sympathetic ganglion cells^{8,10,11}. We tested PC12 cells for responsiveness to ACh in two ways. Microelectrodes filled with 1 M ACh (50–200 M Ω) were positioned just outside the cell to be impaled (within 5 μ m). ACh was kept from diffusing out of the electrode by a 5–8 nA negative holding current (although careful testing with sensitive cells revealed that holding currents of 0–2 nA were sufficient for most electrodes used). When a stable impalement was obtained, discrete pulses of ejecting current between 0.5 and 50 ms duration and up to 50 nA were applied to the ACh electrode. The sensitivity of the PC12 cell was then determined as the amount of depolarisation produced by a given current (expressed as mV nC⁻¹). A second test for ACh sensitivity involved switching the holding current from negative (holding) to positive (ejecting; never more than 9 nA) for several seconds. Sensitive cells depolarised within 1–2 s. Less sensitive cells usually responded almost as fast but less intensely.

PC12 cells grown with NGF for more than 2 weeks responded

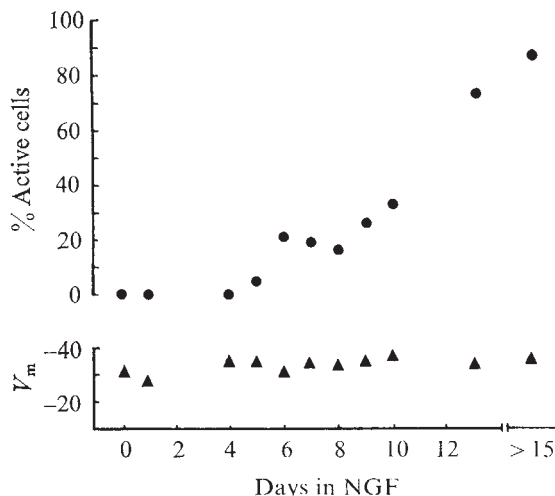


Fig. 2 Percentage of cells in PC12 cultures with action potentials (upper ordinate) and mean membrane potentials (lower ordinate) of these cells (see text) against the no. of days of exposure to NGF. Numbers of impalements for each point were: No NGF, 111; 1 d, 12; 4 d, 22; 5 d, 48; 6 d, 60; 7 d, 77; 8 d, 70; 9 d, 22; 10 d, 18; 13 d, 15; more than 15 d, 42. The standard errors of the mean membrane potentials were between 1 and 3 mV. Note increase in excitability from day 5 to day 13 with no significant change in mean membrane potential.

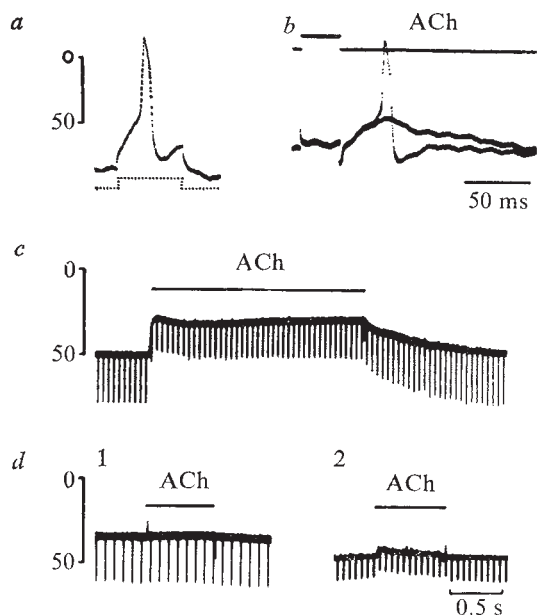


Fig. 3 Response of PC12 cells to iontophoretically applied ACh. *a*, Intracellularly applied depolarising current (dotted line) reaches threshold and triggers an action potential in a NGF-treated PC12 cell. *b*, Two superimposed traces of membrane potential responses to iontophoresis of ACh (14 nA, 30 ms iontophoretic current indicated in top line) on to same cell as in (*a*). Induced depolarisation reached threshold in one trace and triggered an action potential. *c*, Penwriter record of membrane potential of NGF-treated PC12 cell during steady iontophoretic application of ACh ($I = 6.5$ nA). Constant current hyperpolarising pulses were applied to the cell to indicate decrease in input resistance during ACh application. *d*, Penwriter records of ACh responses in two PC12 cells from cultures not treated with NGF. Note no response in (1) ($I = 8.5$ nA) and very small depolarisation in (2) ($I = 6.5$ nA) associated with barely detectable change in input resistance. These records were typical of responses in NGF-treated and non-treated cells. Time calibration in (*d*) applies to *c* and *d*.

to short pulses of current by producing short latency depolarisations which could trigger action potentials (Fig. 3*a,b*). Sensitivity was as high as 100 mV nC^{-1} . The ACh response was also associated with an increased membrane conductance (Fig. 3*c*). The ACh-induced depolarisation became larger with background hyperpolarisation and smaller with background depolarisation of the cell. In the above respects, the response resembles the ACh response seen in chromaffin cells^{7,11,12} and sympathetic ganglion cells¹⁰. Prolonged iontophoretic currents produced depolarisations between 4 and 44 mV (mean of 20 cells, 17 mV) which were also associated with an increased membrane conductance. There were very few cells in the NGF-treated cultures which were unresponsive to ACh. No cells produced hyperpolarising responses.

No cells in the NGF-untreated cultures could be depolarised with short pulses of ACh—even with pulses up to 50 nA in intensity and 50 ms in duration and after hyperpolarisation of the cells to between -50 and -80 mV. Sensitivities as low as 1 mV nC^{-1} would have been detectable. But, PC12 cells grown without NGF could be depolarised by prolonged iontophoretic currents of ACh, but to a much lesser extent than the NGF-treated cells (Fig. 3*d*). The maximum response to several seconds of 8 nA current was 6 mV (mean of nine cells, 4 mV).

The responses to short pulses of ACh in the NGF-treated cells was of short latency (< 5 ms). *d*-Tubocurarine at 10^{-5} M added to the perfusing medium abolished all responses to short pulses of iontophoretic current and substantially reduced the ACh response to prolonged currents. These properties suggest that the ACh response was mediated through nicotinic receptors. This would correlate with the demonstration that nor-adrenaline can be released from PC12 cells by nicotinic agents⁶.

Does inhibition of cell division induce differentiation?

The above data indicate that NGF treatment of PC12 cells profoundly affects their electrical excitability and chemosensitivity. One question raised by these results is whether such changes are merely a consequence of the arrest of cell division which takes place in the presence of NGF, or whether they are due to a more specific action of the factor. To test the former possibility, PC12 cells were treated with the anti-mitotic agents cytosine arabinoside (ara-C, $0.5\text{--}3 \mu\text{M}$) and bromodeoxyuridine (BUDR, $3 \mu\text{M}$) for up to several weeks. While both drugs produced a stable population of non-dividing cells, they did not stimulate fibre outgrowth. Physiological experiments revealed that a low proportion (10%) of such cells could generate active responses (Table 1). These responses were, however, much smaller in magnitude than those displayed by the NGF-treated cells. Furthermore, the ACh sensitivity of the division-arrested cells was comparable with non-NGF-treated rather than NGF-treated cells. Interestingly, cells treated with ara-C maintained their ability to respond to NGF. Such cells extended processes and displayed electrical excitability and ACh sensitivity properties comparable with those of NGF-treated control cells (Table 1).

The above findings suggest that the arrest of cell proliferation alone is not sufficient to account for the effects of NGF on the physiological behaviour of PC12 cells. Such changes are likely to be part of the overall constellation of differentiative events which are triggered by the factor.

Comparison with neuroblastoma cells

Another widely used model culture system with which PC12 cells might be compared are murine C1300 neuroblastoma cells. In appropriate conditions, the latter may also exhibit neuronal features, such as neurotransmitter synthesis, ACh sensitivity and electrical excitability¹. The action potentials of neuroblastoma cells have both Na and Ca components⁹; those of PC12 cells involve predominantly Na. Neuroblastoma cells show hyperpolarising as well as depolarising responses to ACh^{1,13} while the ACh responses of PC12 cells are exclusively depolarising. In addition, PC12 cells have been found thus far to differentiate only in response to NGF (ref. 2), while murine C1300 neuroblastoma cells can be induced to stop dividing and differentiate by a number of different agents but not by NGF (ref. 1). In this regard, the PC12 cells may be analogous to human neuroblastoma cells, which have also been shown to extend processes in response to NGF treatment^{14–16}. PC12 cells are also analogous to cultured human pheochromocytoma cells which also respond to NGF and are electrically excitable^{16,17}.

PC12 line as a model system

The morphology and catecholaminergic properties of NGF-treated PC12 cultures have suggested that the overall effect of the factor on such cells is to promote their differentiation into sympathetic-like neurones^{2,4,16}. The present findings regarding the emergence of electrical excitability and a high level of ACh sensitivity strongly support this contention.

Pheochromocytoma tumour cells are generally considered to be neoplastic counterparts of normal adrenal medullary chromaffin cells. The NGF-treated PC12 cells, in common with both human pheochromocytomas¹⁷ and normal chromaffin cells⁷, can synthesise and store catecholamines, are able to generate action potentials and can exhibit relatively high levels of ACh sensitivity. These properties are also shared with sympathetic neurones. In fact, sympathetic neurones and adrenal chromaffin cells are both of neural crest origin and are both derived from a common stem cell¹⁸. The low degree of physiological development and the ability to take on neuronal properties suggest that the PC12 cells not treated with NGF may possess the characteristics and pluripotency of a primitive, less differentiated common progenitor. If the analogy of PC12 cells to such a

precursor holds, then this, in turn, supports the suggestion^{3,19} that NGF, in addition to its documented effects on post-mitotic cells, has a critical role in promoting the development of normal sympathoblasts into mature, electrically excitable neurones. Thus, the PC12 line may be a unique model system in which to study the differentiation of neuroblasts into neurones as well as for clarifying the role, mechanisms, and consequences of action of NGF on its target cells.

Our results also suggest that the PC12 line should be useful for physiological studies. In particular, it may be feasible to use the PC12 line to obtain biochemical correlates of the development of electrical excitability and chemosensitivity. Seen from a somewhat different orientation, our findings suggest that PC12 cells may be useful to study certain cell-cell interactions. For example, since NGF-treated PC12 cells are highly sensitive to ACh, they should be capable of receiving cholinergic synaptic inputs. Furthermore, not only do PC12 synthesise, store and release catecholamine neurotransmitters, but, like cultured sympathetic neurones²⁰, they may also synthesise, store and release ACh (ref. 21). Thus, PC12 cells may be capable of forming synapses with a variety of target cells as well as with one another.

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Large number hypothesis and continuous creation cosmologies

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The large number hypothesis and the condition that general relativity is satisfied in Einstein units, allows a family of cosmological models, two of which are the Dirac model without creation and the more recent Dirac model with multiplicative creation. The new models have multiplicative creation, a cosmological scale factor $S(t) \propto t^m$, and are spatially flat; a multiplicative steady state model also satisfies the hypotheses. How these models affect the temperature of the Earth and the cosmological deceleration parameter is important.

GRAVITATIONAL and cosmological physics yields three parameters that characterise the macroscopic structure of the Universe—the Hubble constant H , the constant of gravity G , and the average matter density ρ . These parameters are measured in units of mass length and time, a unit independent measure is obtained by comparing them to microscopic parameters of the same dimensions. If m is the mass of an electron, $-e$ its charge, and c the velocity of light, such unit independent measures are

$$\frac{mc^3}{e^2 H} \simeq 6 \times 10^{40}, \quad \frac{e^2}{Gm^2} \simeq 4 \times 10^{42}, \quad \frac{m^4 c^8}{e^6 \rho} \simeq 4 \times 10^{40} \quad (1)$$

These ratios are pure numbers, characteristic of the Universe. They are exceedingly large and approximately equal (the equality could be improved by using other microscopic units).

There are two points of view on these coincidences. One is that physics contains arbitrary parameters. The Universe could be different—it just happens that in our world, these

parameters have their observed values. One argument is^{1,2} that for human life to evolve and ask why the Universe is as it is, these parameters must have values close to those observed; the Universe could be radically different but we would not be here. I feel these arguments are not convincing either at the technical or philosophical level. The second view is that these coincidences are due to some interconnection between microscopic and macroscopic physics. Eddington³ sought to develop a theory of such a casual interconnection, Dirac's^{4,5} approach is to acknowledge that although at present we do not understand this relationship, we can proceed by assuming that for all time

$$\frac{mc^3}{e^2 H} \simeq \frac{e^2}{Gm^2} \simeq \frac{m^4 c^8}{e^6 \rho} \quad (2)$$

and further, that any other large number of the order of 10^{40} is approximately equal to these parameters. Since the first parameter is essentially the age of the Universe in microscopic units all these parameters vary in time but remain equal to each other. This is the large number hypothesis.

Cosmological models and the large number hypothesis

The application of this hypothesis to cosmology restricts the class of possible world models described in atomic units by the Robertson-Walker metric

$$ds^2 = dt^2 - \frac{S^2(t)}{c^2} \left(\frac{dr^2}{1 - kr^2/R_0^2} + r^2 d\theta^2 + r^2 \sin^2 \theta d\phi^2 \right) \quad (3)$$

where $S(t)$ is the scale factor, R_0 a constant length and

$k=0, +1$, or -1 . This metric has at least two characteristic lengths, that are, in principal, empirically determinable (by using atomic clocks and sending light signals between three non colinear co-moving cosmological observers). They are the Hubble length L_H and the radius of curvature of the space $t = \text{constant}$ L_S defined as

$$L_H = \frac{c}{H} = \frac{cS}{\dot{S}}; L_S = \frac{R_0 S}{k}, \quad k \neq 0 \quad (4)$$

Both these lengths are very large and vary as $S(t)$ varies. The large number hypothesis requires that they be proportional to each other, which requires

$$k \neq 0, S = S_0 t + S_1; \quad k = 0, S(t) \propto t^m \text{ or } S(t) \propto \exp(t/t_0) \quad (5)$$

The first result is obvious, since if $k \neq 0$, $\dot{S}$ is constant. As the Universe is observed to be expanding, we can further impose $S_0 \neq 0$ and so S_1 can be absorbed into the zero of time measurement. There are then only two such models, the Milne model⁴ $k = -1$, $S(t) \propto t$, and the recent Dirac⁵ model $k = +1$, $S(t) \propto t$. If $k = 0$, L_S is not a measurable parameter, however, $S(t)$ is not an arbitrary function of t ; to see this we invoke Milne's dimensional argument⁶. Another measurable parameter is the deceleration parameter

$$q = - \frac{S\ddot{S}}{\dot{S}^2} = q(t) \quad (6)$$

This is a pure number. If q varies in time, there must exist a constant t_0 of dimension's time such that $q = q(t/t_0)$. The theory now contains a constant t_0 and a variable $S/\dot{S}$, but the large number hypothesis requires them to be proportional to each other, so either $S/\dot{S} \propto t_0$, $S \propto \exp(t/t_0)$ or $q = S\ddot{S}/\dot{S}^2 = \text{constant}$. The second alternative gives the first solution if $q = -1$, otherwise $S(t) \propto t^m$ where $q = (1-m)/m$.

Turning now to equation (2) the first and second terms give $G \propto H$ so that in general G varies in time as measured in atomic units. The first and third conditions give $\rho \propto H$, but as the Universe expands, the number of particles in a co-moving volume $N_0 \propto \rho S^3$, hence $N_0 \propto HS^3$ which is general varies with time. Particles must, therefore, be created or annihilated, the exception being the original Dirac model $S(t) \propto t^{\frac{1}{3}}$. In Table 1 we list the Robertson-Walker models satisfying the large number hypothesis.

Table 1 Cosmological models satisfying the large number hypothesis*

Model	k	$S(t)$	H	G	N_0
Milne	-1	t	$1/t$	$1/t$	t^2
Dirac	+1	t	$1/t$	$1/t$	t^2
Steady State	0	$\exp(t/t_0)$	$1/t_0$	const	$\exp(3t/t_0)$
Power Law	0	t^m	m/t	$1/t$	t^{3m-1}
Original Dirac	0	$t^{\frac{1}{3}}$	$1/3t$	$1/t$	const

*The three models $S(t) = 1$, $k = 0, +1, -1$, are ruled out by the condition $H \neq 0$.

Field equations in Einstein units

To advance any further, it is necessary to impose some additional constraints on the theory. First, where is the matter created? In the standard steady state theory^{7,8}, the newly created matter is supposed to be created uniformly throughout space and time. Dirac has recently proposed that creation is multiplicative⁹—that is, new matter is created in proportion to existing matter; we shall accept this hypothesis. The empirical

success of general relativity further led Dirac to hypothesise that this theory must remain valid for macroscopic physics, due allowance being made for the creation of matter, and was led to deduce the new model described above, $S(t) \propto t$, $N_0 \propto t^2$, we shall also retain this hypothesis; however, it hardly restricts the possible cosmological solutions, only the Milne model does not satisfy this constraint.

Since general relativity is to describe macroscopic gravitational physics, we work in Einstein units in which the macroscopic mass mN_0 , the constant of gravity G , and the velocity of light c are constant. All we are doing here is to define as our unit of mass as (say) the mass of the sun, our unit of time is defined by a gravitational clock (say) the orbital period of the Earth, and the unit of length is defined from the unit of time and a defined constant velocity of light. In this set of macroscopic units, Einstein's equations

$$R_{ij} - \frac{1}{2}g_{ij}R + \Lambda g_{ij} = \frac{8\pi G}{c^2} T_{ij} \quad (7)$$

are valid. However, since matter is being created, the number of elementary particles in the sun varies in time, so, too, do the mass m and charge e in these gravitational units, subject to the condition that mN_0 is constant.

If τ and ρ are time and distance in Einstein units, then if the Robertson-Walker metric

$$ds_E^2 = d\tau^2 - \frac{S^2(\tau)}{c^2} \left(\frac{d\rho^2}{1 - k\rho^2/R_0^2} + \rho^2 d\theta^2 + \rho^2 \sin^2\theta d\phi^2 \right) \quad (8)$$

is introduced into the field equation (7), we obtain the usual family of cosmological models. However, most of these are incompatible with the large number hypothesis, introducing two or more cosmological lengths that are not proportional to each other. The only admissible solutions are those that satisfy equation (5) of which there are just two.

Einstein models:

$$S = 1, k = +1, \Lambda = \frac{1}{R_0^2} = \frac{4\pi G\rho}{c^2}, \rho, \text{ constant} \quad (9)$$

Einstein-de Sitter model:

$$S \propto \tau^{2/3}, k = 0, \Lambda = 0, \rho S^3 = \rho_0, \text{ a constant} \quad (10)$$

Large number hypothesis in Einstein units

The large number hypothesis shown by equation (2) is, of course, independent of any system of units. In Einstein units with G , c and ρ_0 constant this requires

$$H \propto \frac{m^3}{e^4}, \quad \frac{e^8}{m^8} \propto S^3, \text{ hence } H \propto \frac{1}{S^{3/2}} \quad (11)$$

The Hubble constant H is not S'/S (the dash denotes differentiation w.r.t. τ). It is defined, and measured, as the constant of proportionality in the red shift luminosity distance relation. Besides the usual contribution due to expansion in τ time, there is a further contribution due to the change in frequency of a particular atomic transition as e and m vary in τ time, this frequency

$$\nu \propto \frac{m}{e^2} \propto \left(\frac{1}{m^2(\tau)S^3(\tau)} \right)^{1/4} \quad (12)$$

The ratio of the frequency of radiation from a given transition in a distant source at time τ_1 , to the frequency of radiation

from the same transition as an observer is, therefore

$$\frac{\mu_1}{\mu_0} = \frac{S(\tau_0)}{S(\tau_1)} \left(\frac{m^2(\tau_1) S^3(\tau_1)}{m^2(\tau_0) S^3(\tau_0)} \right)^{1/4} \quad (13)$$

The source is at co-moving Einstein coordinate $(\rho_1 \tau_1)$, the receiver at $(0, \tau_0)$, since the signal propagates along null geodesics we have

$$\int_0^{\rho_1} \frac{d\rho}{1 - k\rho^2/R_0^2} = \int \frac{cd\tau}{S(\tau)}, \quad \tau_1 = \tau_0 - \rho \frac{S(\tau_0)}{c} + \dots \quad (14)$$

and hence equation (13) becomes

$$\frac{v_1}{v_0} = 1 - \left(\frac{1}{4} \frac{S'}{S} - \frac{1}{2} \frac{m'}{m} \right) \rho_1 \frac{S(\tau_0)}{c} + \dots \quad (15)$$

Now ρ_1 is the coordinate distance, not the luminosity distance l_1 , to leading order, however $l_1 = \rho_1 S(\tau_0)c$; to second order this relation is not valid due to the change in luminosity of the source in τ time, the change of $S(\tau)$, the curvature of spaces $\tau = \text{constant}$ and the creation of photons in the beam of radiation as it travels from the distant source to the receiver. These effects will be considered below when estimating the deceleration parameter for these models. To leading order the red shift luminosity distance relation is

$$cz = Hl, \quad H = \left(\frac{1}{4} \frac{S'}{S} - \frac{1}{2} \frac{m'}{m} \right) \quad (16)$$

We can now combine this result for the Hubble constant in Einstein units with the result (11) $H \propto S^{-3/2}$ to obtain the two solutions that satisfy the large number hypothesis and Einstein's equation in Einstein units

Einstein Universe:

$$S = \text{constant}, H = \text{constant}, m \propto \exp(-\tau/\tau_0) \\ N_0 \propto \exp(\tau/\tau_0), e \propto \exp(3\tau/4\tau_0) \quad (17)$$

Einstein-de Sitter Universe:

$$S \propto \tau^{2/3}, H \propto \tau^{-1}, m \propto \tau^{-n}, N_0 \propto \tau^n, e \propto \tau^{(1-3n)/4} \quad (18)$$

where $n \neq -1/3$ but is otherwise arbitrary ($n = 1/3$ gives $H = 0$).

The cosmological models in atomic units

Having obtained the equations (17) and (18) in Einstein units we can transform them back to the more familiar atomic units (AMU) in which e, m, c are constant but G and ρ_0 are not. (We could have scaled Einstein's equations in Einstein units to obtain their equivalent form in atomic units by the conformal transformation $g_{ij} \rightarrow \beta g_{ij}^*$, and worked throughout in AMU: the results would, of course, be the same. However, we prefer to use Einstein as this emphasises the assumptions, and simplifies the analysis.) The AMU of length and time

$$l_A \propto \frac{e^2}{m} \propto (m^2 S^3(\tau))^{1/4} \quad (19)$$

varies in Einstein units but is by definition constant in AMU. All lengths and times in Einstein units must, therefore, be scaled by this factor to obtain their values in AMU, so that if t is atomic time

Einstein model:

$$dt \propto (m^2 S^3)^{-1/4} d\tau \propto \exp(\tau/\tau_0) d\tau \quad (20)$$

Einstein-de Sitter model:

$$dt \propto (m^2 S^3)^{-1/4} d\tau \propto \tau^{(n-1)/2} d\tau \quad (21)$$

These integrate to give

Einstein model:

$$t \propto \exp(\tau/\tau_0) \quad (22)$$

Einstein-de Sitter model:

$$t \propto \tau^{(n+1)/2} \quad n \neq -1 \quad (23)$$

$$t \propto \ln \tau / \tau_0 \quad n = -1 \quad (24)$$

Similarly, the metric in AMU is obtained by scaling the metric in Einstein units

$$ds_A^2 = \frac{ds_E^2}{(m^2 S^3)^{1/2}}$$

yielding

Einstein model:

$$ds_A^2 = \exp(\tau/\tau_0) \left(d\tau^2 - \frac{1}{c^2} \left(\frac{d\rho^2}{1 - \rho^2/R_0^2} \right) + \right. \\ \left. + (\rho^2 d\theta^2 + \rho^2 \sin^2 \theta d\phi^2) \right) \quad (25)$$

Einstein-de Sitter model:

$$ds_A^2 = \tau^{n-1} \left(d\tau^2 - \frac{1}{c^2} \left(\frac{\tau}{\tau_0} \right)^{4/3} (d\rho^2 + \rho^2 d\theta^2 + \rho^2 \sin^2 \theta d\phi^2) \right) \quad (26)$$

which gives the three solutions: the Dirac Creation Model (DC), the power law models (PL) and the Steady State theory with multiplicative creation (MSS)

$$\text{DC: } ds_A^2 = dt^2 - \frac{1}{c^2} \left(\frac{t}{t_0} \right)^2 \left(\frac{dr^2}{1 - r^2/R_0^2} + r^2 d\theta^2 + r^2 \sin^2 \theta d\phi^2 \right), \\ N_0 \propto t^2, G \propto 1/t \quad (27)$$

$$\text{PL: } ds_A^2 = dt^2 - \frac{1}{c^2} \left(\frac{t}{t_0} \right)^{2m} (dr^2 + r^2 d\theta^2 + r^2 \sin^2 \theta d\phi^2), \\ N_0 \propto t^{3m-1}, G \propto 1/t \quad (28)$$

$$\text{MSS: } ds_A^2 = dr^2 - \frac{1}{c^2} \exp(2t/t_0) (dr^2 + r^2 d\theta^2 + r^2 \sin^2 \theta d\phi^2), \\ N_0 \propto \exp(3t/t_0), G = \text{const} \quad (29)$$

Of the possible solutions listed in Table 1, only the Milne model $S \propto t, k = -1$ is excluded. The condition that the large number hypothesis be satisfied and that Einstein's equations be satisfied in Einstein units (G, c, ρ_0 constant) does not lead to a unique cosmological model, but to two special cases, the Dirac creation cosmology and the steady state cosmology with multiplicative creation, and to a family of power law models $S(t) \propto t^m$. Indeed, even the Dirac creation cosmology is not unique since R_0 in the equation (27) is arbitrary. Only by introducing a theory of the creation process can we expect to narrow down the range of possible models, for example, if there has been no creation or annihilation of matter since the 'origin' of the Universe, the only viable model is the original Dirac model without creation $S(t) \propto t^{1/3}$. However, I see no metaphysical attraction in such a hypothesis, and in the absence of any empirical evidence, there is no way to differentiate between the models.

The black body temperature of the Earth

For all the models derived here, we can calculate the variation of the thermal equilibrium temperature of the Earth during its

lifetime. This is not, however, necessarily the actual thermal history of the Earth since many factors, such as atmospheric composition, and albedo need to be considered, but it should give some indication of the consequences of these models for the terrestrial environment. The flux of radiation falling on the Earth varies for several reasons, (1) the Sun's luminosity is sensitively dependent on the value of G , and the mass of the Sun, (2) the Earth's orbit spirals outwards (or inwards) as the mass of the Sun and Earth and G vary, (3) the Sun evolves chemically. We shall neglect (3), at constant mass and G this corresponds to an increase in solar luminosity of about 35%.

The luminosity of a star (L) of about solar mass depends on its mass M and the constant of gravity G approximately as $L \propto G^7 M^6$. Working in AMU we find that the equilibrium temperature

$$T_e \propto \left(\frac{L}{r_A^2}\right)^{1/4} \propto \left(\frac{G^7 N_0^6}{r_A^2}\right)^{1/4} \quad (30)$$

where r_A is the radius of the Earth's orbit in AMU. Provided the newly created matter is created with the same velocity as its surroundings, thereby not exerting a drag on the Earth's orbit⁹, the radius of that orbit is constant in Einstein units. Its value in atomic units is simply obtained by scaling by the factor $(m^2 S^3)^{1/4}$ of equation (11); using the results in equations (27) to (29) gives

$$\text{DC: } T_e \propto t^{1/4}, \text{ PL: } T_e \propto t^{(9m-8)/4}, \text{ MSS: } T_e \propto \exp\left(\frac{9}{4} Ht\right) \quad (31)$$

The estimated value of the Hubble constant is about $5 \times 10^{-11} \text{ yr}^{-1}$, and the age of the Earth is about $5 \times 10^9 \text{ yr}$, hence the ratio of equilibrium temperature today to when the Earth was formed is

$$\text{DC: } \frac{T_e}{T_0} \propto \left(\frac{4}{3}\right)^{1/4}, \text{ PL: } \frac{T_e}{T_0} \propto \left(\frac{4m}{4m-1}\right)^{(9m-8)/4}, \quad (32)$$

$$\text{MSS: } \frac{T_e}{T_0} = \exp\left(\frac{9}{16}\right)$$

Inasmuch as any conclusion can be drawn from climate studies, the flux of radiation falling on the Earth has increased. Indeed, if it was ever less than 90% of its present value, the Earth would now probably be completely ice covered¹⁰. This is a problem for standard theory which predicts a solar constant some 70% or so of the present value during the early history of the Earth¹¹. If $m < 8/9$ the power law models yield a decreasing solar flux. (These models would not, however, explain the low neutrino flux since the evolutionary lifetime of a star $\propto L/M \propto t^{12m-11}$ so that such models would be more evolved than the standard model giving an increased solar neutrino flux.

The cosmological deceleration parameter

As the intrinsic luminosity of a star varies as the Universe evolves, so too will the intrinsic luminosity of a galaxy. This 'evolutionary effect' will manifest itself in the observed departures from the Hubble relation, through the deceleration parameter. While a detailed analysis requires a study of stellar and galactic evolution in these cosmological models, some idea of the observational consequences is obtained by assuming $L_{\text{galaxy}} \propto L_{\text{star}} \propto G^7 M^6$.

Consider a galaxy at coordinate distance r_1 , the energy per unit area received at the origin of coordinates at time t_0 (measured in atomic units) is¹²

$$P = \left(\frac{L(t_0)}{4\pi S^2(t_0) r_1^2} \right) \left(\frac{N_0}{N_1} \right) \quad (33)$$

where $L(t_1)$ is the luminosity of the source at the time of

emission t_1 , and the extra factor $N_0 N_1$ is due to the multiplicative creation of photons in the beam on its journey from source to receiver. The coordinate distance r_1 satisfies

$$\int_{r_1}^{r_0} \frac{dr}{1 - kr^2/R_0^2} = \int_{t_1}^{t_0} \frac{cdt}{S(t)}, \quad r_1 = \frac{zc}{S(t_0)} \left(1 - \frac{1}{2}(q_0+1)z + \dots\right) \quad (34)$$

where z is the red shift of the source $(1+z) = S(t_0)/S(t_1)$, and $q_0 = -S\ddot{S}/\dot{S}^2$ is the deceleration parameter at $t=t_0$. Equations (33) and (34) yield the magnitude redshift relation

$$m_g = -2.5 \log\left(\frac{L(t_1)}{L(t_0)}\right) + 5 \log z + 2.5 \log(1+z) - 5 \log H + \text{const} \quad (35)$$

This relation is valid for all the models considered here, the value of m does not appear explicitly as the term

$$\frac{N_0}{N_1} = \left(\frac{S(t_0)}{S(t_1)}\right)^{(3m-1)/m} \simeq \left(1 + \frac{3m-1}{m} z + \dots\right) = (1 + (2-q_0)z + \dots) \quad (36)$$

and the terms involving q_0 exactly compensate. The same is true for the MSS model. Neglecting galactic evolution, all these models have an effective deceleration parameter $q_e = +2$, the particular characteristics of the model only enter through the evolution of the galaxy. If we assume that $L_{\text{galaxy}} \propto L_{\text{star}}$, then for the power law models

$$\frac{L(t_1)}{L(t_0)} = \left(\frac{t_1}{t_0}\right)^{(15m-12)} = \left(\frac{S(t_1)}{S(t_0)}\right)^{(15m-12)/m} \left(1 + \frac{15m-12}{m} z + \dots\right) \quad (37)$$

and again this result holds for the MSS model with $m \rightarrow \infty$. Including this estimate of the evolutionary effect, we find an effective deceleration parameter

$$q_e = 2 - \left(\frac{15m-12}{m}\right) = \frac{12-13m}{m} \quad (38)$$

For the DC model $q_e = -1$, for the original Dirac model without creation $m=1/3$, $q_e=23$, for the MSS model $q_e = -13$, for the constant Earth temperature model $m=8/9$, $q_e=0.5$. It would appear that there are models whose predictions are not incompatible with observation.

Conclusions

Assuming the validity of the large number hypothesis, multiplicative creation, and that general relativity is satisfied in Einstein units, we find a family of possible cosmological models. Two of these are the original Dirac model without creation and the new Dirac model with creation, but other solutions exist with $S(t) \propto t^m$, m arbitrary, and a multiplicative steady state theory. Further restrictions have to be imposed on the theory before this class of solutions can be narrowed down to one model.

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letters to nature

A line feature at 64 keV in the X-ray spectrum of Her X-1

TRUMPER and his colleagues¹ have reported observations which give evidence of a hard X-ray line feature in the spectrum of the pulsed flux of Her X-1. They suggest that this is cyclotron radiation from the poles of a neutron star. We discuss here some similar measurements made by the Imperial College Scintillation Telescope (ST) on Ariel 5 during a visit to the Hercules region, scheduled many months in advance of the observation. The binary system Her X-1 is known to emit X rays for at least 10 d in its 35-d cycle and our measurements were made during the ON period 3–13 February 1977.

The ST covers the energy range 26 keV to 1.2 MeV and consists of a CsI(Na), actively collimated detector crystal of 8 cm², offset from the satellite spin axis by 3°. Further details of the instrument and our observational technique can be found elsewhere². We observed Her X-1 with the ST instrument in spectral mode for 2 d on the rising side of the light curve and 4 d on the falling side. This data has a time resolution of 8 min and thus we can only discuss the time averaged spectrum. Data from both epochs have been summed to obtain the best statistics and the resulting pulse height spectrum is shown in Fig. 1. All upper limits are 2 standard deviations (s.d.). It is clear from Fig. 1 that there is a significant flux excess in the 56–94 keV band.

To deconvolute this data from the detector response, a three-component spectrum was used consisting of a thermal bremsstrahlung component of $kT \sim 4$ keV, a line component at a variable energy, and a hard, power-law tail consistent with our upper limits. This gives the most conservative estimate for the significance of the line feature in the data. The relative intensities of the three components were varied, folded through a

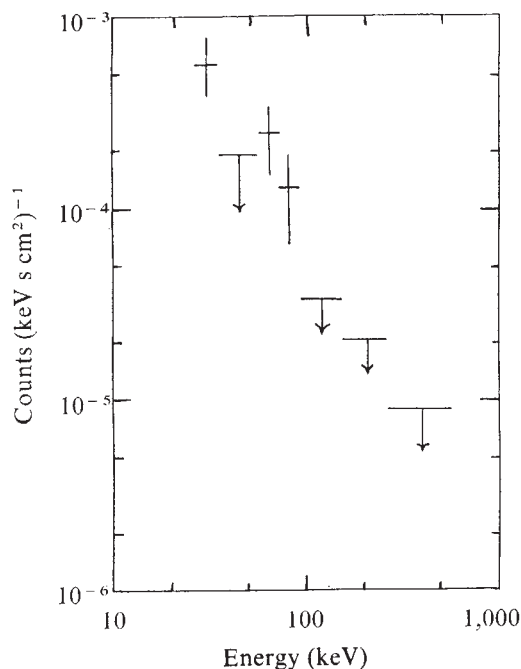
detector interaction matrix (as explained in ref. 2) and compared with the pulse height spectrum until a best fit was achieved. The fit was constrained to lie immediately below all the 2 s.d. upper limits. This spectrum was then used to deconvolute our data points and the result is shown in Fig. 2. Again all upper limits are 2 s.d. We believe that any spectrum consistent with the points shown in Fig. 2 would produce the pulse height spectrum observed. The fitted spectrum of Boldt *et al.*³ from rocket flight data has also been shown in Fig. 2. Their absolute flux has been reduced by a factor of two to fit with our point at 30 keV. This fitting has no effect on the intensity of the line at 64 keV due to the steep nature of this part of the spectrum.

Assuming the spectral feature evident in Fig. 2 is the result of a narrow line, we can set limits to its position by considering the detailed spectral response of our channels. This yields an energy of 64 ± 6 keV at 1 s.d. Alternatively, by assuming a broader line centred near the mid-point of our channel, we can find an upper limit for its FWHM. This gives 16 keV at 1 s.d. Our estimate for the line intensity is 0.017 ± 0.007 photons cm⁻² s⁻¹ and this value is relatively insensitive to variations in the assumed underlying spectrum used for unfolding the results. The error is calculated for counting statistics only. Separate analysis of data from the rising and falling side of the X-ray light curve both yield evidence for the line feature.

The observations of February 1977 can be compared with a previous shorter observation⁴ made by the same instrument in March 1975 during the early part of the ON phase. Identical analysis of these earlier data reveal a flux in the 26–34 keV band which is three times higher than the new flux. But no spectral feature is apparent at 64 keV and there is only a 2% probability that the line feature was also three times brighter than the new intensity. A line of intensity equal to the one reported here would not have been detectable due to the shorter observation time.

To explain the line, consider the usual model for X-ray emission from Her X-1 as being due to the infall of material near the surface of a neutron star after pushing its way down the polar funnels of the intense magnetic field. Correcting the line energy for gravitational red-shift (taking the origin of emission as 1.2×10^6 cm for a $1M_{\odot}$ object) yields 73.1 keV as the initial energy. Classical cyclotron emission theory gives $h\nu_0 = (eHh/2\pi m_0 c) \times \sqrt{1-\beta^2}$ for the energy of first harmonic emission in a field H where β is the electron velocity expressed as a fraction of c and m_0 is its rest mass. This leads to a field strength estimate of $H = 7.3 \times 10^{12}$ G. At this field strength, however, the De Broglie wavelength of a 73 keV electron is four times its cyclotron radius and a relativistic, quantum mechanical treatment would seem more appropriate. Johnson and Lippmann⁵ find total energy eigenvalue solution to Dirac's equation given by $E = \pm [c^2 p_{\perp}^2 + m_0^2 c^4 + e\hbar c H (2n + s + 1)]^{1/2}$, where $s = \pm 1$, the electron spin state, n is the principal quantum number and $p_{\perp}$ is momentum parallel to H . Supposing that both parallel and anti parallel spin states arise while the material is falling into the polar funnels, $s = +1 \rightarrow s = -1$ transitions may be expected to occur, leading to energy release ΔE where $E_i^2 - (E_i - \Delta E)^2 = 2e\hbar c H$. E_i , the initial energy, is estimated as the sum of rest mass energy plus energy in the spin-field interaction plus thermal energy and we take the last of these contributions as 13 keV, based on a previous data fit of Carpenter *et al.*⁴. A value $H = 6.9 \times 10^{12}$ G results. Lodenquai *et al.*⁶ find various allowed angular momentum states in a more complete, but non-relativistic treatment. Use of their work and the assumption of angular momentum transitions would lead to a field value roughly comparable with

Fig. 1 The pulse height spectrum (converted to an energy scale) obtained from Her X-1 with the hard X-ray detector on Ariel 5 by combining data from the periods 4–6 and 9–13 February 1977.



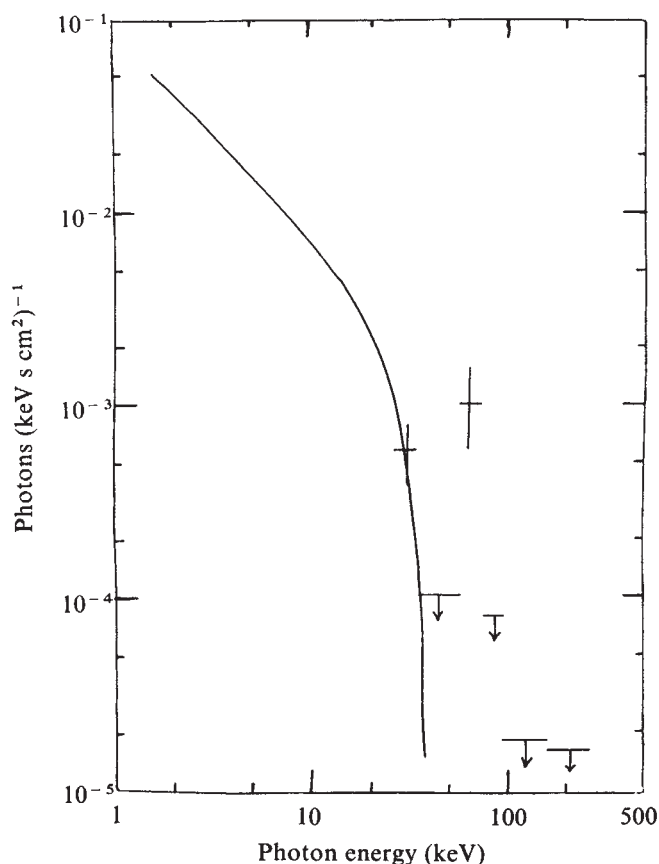


Fig. 2 The spectrum of Fig. 1 unfolded as described in the text. Also shown, as a continuous line, is the best fit spectrum from the proportional counter data of Boldt *et al.*³ reduced in absolute intensity by a factor of two.

the above estimates. Note that a line arises naturally in the quantum mechanical treatment, rather than the wide spread implied by the $\sqrt{(1-\beta^2)}$ factor in the classical treatment. In fact, putting $\Delta E \propto \Delta H$ and assuming a dipole field implies an emitting region depth 8% of a neutron star radius in size if we take our observations of the line width. Also note that high electron kinetic energies and, by implication, X-ray continuum radiation at appreciable levels around 100 keV are not necessary. A classical treatment of the cyclotron self-absorption effect suggests that for the observed flux values, escape of the X rays considered is not a problem.

An alternative explanation in terms of radioactive decay of nuclides produced by explosive nucleosynthesis during neutron star formation more than 10^4 years ago is less promising. We use Clayton's⁷ estimates of supernovae yields required to give the ^{60}Ni Solar System abundance, with 50% produced via the ^{60}Fe progenitor, which decays with a 3×10^6 -yr half life. The ^{60}mCo decay to ground state within this chain is via a 58.6-keV (~ 50 keV after redshift) line, provided the internal conversion branch in atomic Co is inhibited by the degenerate gas conditions at the neutron star surface. A low Her X-1 distance estimate of 600 pc is then required to get $\sim 4 \times 10^{-5} \text{ cm}^{-2} \text{ s}^{-1}$ photons at the Earth, well short of the observed flux. Furthermore, a magnetospheric controlled screening process would need to be invoked to explain the occurrence of the line in the pulsed component and this would further decrease the predicted flux. No other process seems as likely as the one discussed. But a lack of temporal variation in the line flux would perhaps favour an explanation in terms of radioactivity, in which case the estimates for nuclide production from supernovae must be considerably revised.

A search for the equivalent proton cyclotron transition at 274 Å (after redshift) would be interesting. Radiation from

such a line is likely to be highly perturbed by the surrounding plasma, however. If our explanation of the observations is correct, a new way of exploring pulsar magnetic fields is opened up.

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Relation between metallicity and multiplicity for solar type stars

It has been suggested that the ratio of heavy elements to hydrogen (metallicity) in a contracting interstellar cloud might affect the process of star formation. There is now statistical evidence for solar type stars that the metallicity of a forming stellar system is correlated with the multiplicity of the resultant system and may actually determine whether the system will be multiple or single.

The most direct and complete data on the multiplicity of solar type stars are given by Abt and Levy¹. The δm_1 index^{2,3} is thought to provide rather precise determinations of the relative metallicities of late F dwarfs and subgiants. Lindemann and Hauck⁴ give photometric data sufficient to calculate δm_1 (β) for 87 of Abt and Levy's 135 stars, and $\delta m_1(b-y)$ for 27 more. Two of the stars must be omitted because they are too cool for β or $b-y$ to be reliable. Of the 112 remaining stars with available δm_1 , Abt and Levy (their Table 2) describe 15 as having a 'probable' or 'possible' constant or variable velocity. Omitting these slightly uncertain cases from consideration, there are 97 stars with reliable values of δm_1 and definite statements whether their radial velocities are constant or not. The latter are interpreted as multiple systems while the former may be single stars, stars with low mass secondaries and/or extremely long periods, or close binaries viewed pole on.

The 97 stars divide into three categories of metallicity: (1) $\delta m_1 \leq 0.000$, the mean metallicity of the Hyades cluster stars or higher; (2) $0.000 < \delta m_1 \leq 0.030$, metallicities ranging from half that of the Hyades stars up to that of the Hyades stars; (3) $\delta m_1 > 0.030$, metallicities less than half that of the Hyades stars.

There are seven stars in the metal-rich group (1): six have a variable velocity (interpreted as multiple); one has a constant velocity and a companion with a common proper motion (CPM) and is interpreted here as a multiple system. None has a constant velocity and no known or suspected companion (interpreted as 'single?').

There are 74 stars in group (2): 35 have a variable velocity (multiple); nine have a constant velocity and a CPM companion or are known members of a visual binary system (multiple); 30 have constant velocities and no known or suspected companions (single?).

There are 16 stars in the moderately low metallicity group (3): one has a variable radial velocity (multiple); two have constant velocities and either has a CPM companion or is a known member of a visual binary (multiple); 13 have constant velocities and no known or suspected companions (single?).

Table 1 Multiplicity for three categories of metallicity

	$\delta m_1 \leq 0.000$		$0.000 < \delta m_1 \leq 0.030$		$\delta m_1 > 0.030$	
	Multiple	Single	Multiple	Single	Multiple	Single
High velocity	0	0	3	3	0	5
Low velocity	7	0	41	27	3	8
Combined	7	0	44	30	3	13

Eleven of the 97 stars are intermediate (not extreme) 'high velocity' stars with space motions in excess of 63 km s^{-1} . It has long been known that high velocity stars tend to be single and also that they tend to have low metallicity. Table 1 emphasises that a metallicity-multiplicity correlation exists within both the high velocity and low velocity groups. In other words, it is a phenomenon of both population 1 and population 2 stars. (It is important to determine whether the unresolved combination of the colours of two members of a close binary will produce an artificially small δm_1 , leading to a false interpretation that the pair is richer in heavy elements than they actually are. Calculations of various combinations of the observed colours of apparently single, constant velocity stars suggest that this is not the case.) We conclude that the chemical composition of the star forming cloud is closely related to the probability of forming a multiple system.

There is new evidence from spectral quantification⁵ and from spectral classification⁶ that the Sun has the same metallicity as the mean of the Hyades cluster stars. This result, acceptance of a metallicity-multiplicity correlation, and the awareness that the internal scatter in the δm_1 values is about 0.01 magnitudes, suggest that all solar type stars of $\delta m_1 \lesssim 0.020$ (which is about $\frac{2}{3}$ the solar metallicity or greater) are multiple systems with the observed constant velocity systems being either systems viewed pole on or systems with low mass secondary stars or planets. This inference is consistent with Abt and Levy's conclusion that single stars are rare among low velocity stars.

The possibility also exists that all solar type systems of whatever metallicity form as multiples with the lower metallicity systems (including population 2) tending to have large mass ratios and being more likely to have planets as secondaries. Carried to the extreme, this speculation implies that the hypothetical population 2 planetary systems might have given rise to life more than 5,000 Myr before our Sun formed.

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Cosmic rays and ancient catastrophes

A NUMBER of suggestions have been made¹⁻³ that encounters of the solar system with extra-solar phenomena in earlier epochs led to climatic and biological catastrophes. Most recently Whitten *et al.*⁴ and Clark *et al.*⁵ have drawn attention to the role of nearby supernovae (within $\approx 10 \text{ pc}$) from which catastrophic effects are expected (primarily the depletion of the atmospheric ozone layer and consequent transmission of damaging solar ultraviolet.) Whitten *et al.* estimate the probability of an encounter within a period of 10^8 yr to be 1-5% and Clark *et al.* calculate 50% for the same quantity. In this letter, we argue that it is likely that rare and very big solar flares, and their attendant cosmic-ray fluxes, will give even more dramatic effects. This idea is not new, in that Reid *et al.*⁶ have drawn attention to the effect of solar flares on the evolu-

tion of life, but here we endeavour to estimate the likely frequency of solar flares of sufficient strength to have significant effects. Furthermore, we draw attention to the 'explosion' at the galactic centre postulated by some⁷ to have occurred about 10^7 yr ago; if this actually took place then its effect too would have been important and exceeded likely supernova phenomena.

In view of the fact that the Sun is the nearest active astronomical object, it is clearly necessary to examine its activity characteristics in detail before going further afield. There is a wealth of data on solar flares and, for the past 35 yr, cosmic rays from the Sun have been recorded, with an increasing degree of completeness. Measurements have been made of particles at ground level, at a variety of altitudes and, recently, outside the atmosphere with satellites. In each case, data have been collected at various geomagnetic latitudes. We have summarised the published data (most of it relevant to high galactic latitudes where the geomagnetic cut off is small) in Fig. 1. The energy density received at the top of the atmosphere is taken as the relevant parameter (defined as integrated energy in the flare for particles above a threshold energy per unit area: erg cm^{-2}) and its frequency distribution is taken for two periods (1956-1960)⁸ and (1961-1972)⁹. The fluxes for 1956-1960 refer to a threshold of 100 MeV and the corresponding fluxes for protons above 20 MeV will be higher by a factor of the order of 5. A comment is necessary about the magnitude of the threshold energy relevant to the present analysis. A prominent effect is the destruction of the ozone

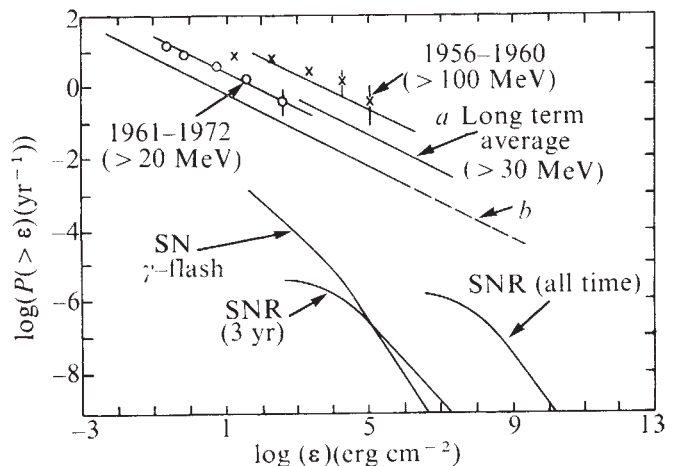


Fig. 1 Integral frequencies of solar cosmic-ray bursts at the Earth. The abscissa is the energy density in the burst and relates to the top of the atmosphere. Line *a* is a rough estimate of the long period average burst frequency (for energies above $\sim 30 \text{ MeV}$) and is derived from measurements made on protons during the very active period 1956-1960 and during the most recent solar cycle 1961-1972 (the latter being of apparently rather average solar activity as judged by the mean sunspot numbers). Most of the particles under consideration normally arrive in the polar regions. Line *b* represents the frequency distribution when the burst energy density is averaged over the Earth's surface. SN γ -flash denotes the frequency distribution of energy deposition from the γ -ray flash from supernovae (using the result of Clark *et al.* 1977, for a supernova at 10 pc). SNR (3 yr) and SNR (all time) represent energy deposition over a 3-yr period, and integrated over the whole time, respectively, from protons when the Earth is immersed in a supernova remnant. *P* is probability and ϵ the energy density.

layer by NO produced after ionisation of the stratosphere¹⁰ and the incident proton energy necessary to reach the appropriate levels in the stratosphere is ≈ 30 MeV. Thus, an energy threshold of 20 MeV is only a little low for the necessary limit.

Two features are apparent in Fig. 1: there is a large difference in the absolute frequencies and the slopes of the variations are very similar in the two cases. The disparity in absolute frequencies relates to the difference in general solar activity in the two periods. The interval 1961–1972 covers the most recent solar maximum period together with the previous solar minimum and 1956–1960 comprises the corresponding previous solar maximum. The mean sunspot numbers are relevant; Allen¹¹ quotes $R_m = 110.6$ for the solar maximum in 1968.9 and $R_m = 201.3$ for 1957.9 to be compared with an overall average $R_m \approx 112$ for the last 270 yr, the period over which reliable measurements have been made. There is, thus, some evidence for the frequency of bursts of solar cosmic-ray particles arriving at earth being strongly dependent on mean sunspot number. It is interesting that an analysis by Schöve¹² suggests that only two periods, centred on 1372 and 1558 had similarly high sunspot numbers to 1956–1960. Thus, activity on the scale of 1956–1960, although rare is almost certainly not unique.

Remembering that the 1956–1960 data refer to energies above 100 MeV and that the contributions to $P(>\epsilon)$, probability ($>$ energy density), from the different cosmic ray flare magnitude ranges are additive we estimate that the very long term average for $P(>\epsilon)$ and proton energies above 30 MeV is at least as high as line *a* in Fig. 1. There seems to be evidence for an empirical power law for $P > \epsilon$ over seven orders of magnitude and we feel that this can probably be extended by at least several magnitudes more.

The region of interest with respect to supernovae is indicated in Fig. 1 by the line 'SN— γ flash'. The energy density of 10^6 erg cm⁻², corresponding to one event in 10^8 yr, is the value adopted by Clark *et al.* for γ rays from a supernova explosion at a distance of 10 pc and we have calculated the values for various distances to give the line. The interesting energy region considered by Clark *et al.* (10^6 erg cm⁻²) is only a little way beyond the highest detected flare energy and extrapolation over this small energy range can surely be made. The extrapolation of line *a* itself is not immediately relevant, however, because the effective area over which the indicated energy density acts is not the full area of the earth, the reason being the shielding introduced by the Earth's magnetic field. As a very conservative figure we assume that the low energy particles in question arrive only at geomagnetic latitudes above 70°, where the average geomagnetic cut-off rigidity is only ~ 0.15 GV (it is permissible to use this value for a proton energy threshold of 20–100 MeV because the large cosmic-ray flares have rather flat energy spectra at energies below 200 MeV, or so). The operative area is $\approx 3\%$ of the total. Using this factor the effective density averaged over the whole Earth is as shown in line *b*.

Another phenomenon is also relevant: the reversing, during past epochs, of the Earth's magnetic field⁶. During the period when the field is small, and the cutoff rigidity likewise, the flare particles will blanket the Earth completely. A very approximate analysis gives an effective average again not far from line *b* (we are indebted to C. J. Hatton and R. W. Girdler for discussions on these points).

Figure 1 also indicates that significant solar flare effects (similar to SN at 10 pc) will be expected about every 10^3 yr—much more frequently than expected for close supernovae—and, indeed, considerably stronger effects are likely on a time scale of 10^5 yr.

An important question is whether there is supporting evidence from other stars for the emission of energetic flares (and cosmic rays from them). Although in many solar flares the particle energy is only $\approx 1\%$ of the total flare energy, in the largest cosmic ray flares, the particle energy seems to approximate to the optical energy¹³ ($\approx 10^{32}$ erg). Thus, the extrapolation

is only to some 10^{34} erg or so in total energy in order to be well inside the SN region. There is little doubt that stellar flares do give cosmic-ray energies in this region. Lovell¹⁴ has summarised the evidence for cosmic rays from M- and K-stars and shown that their flare energies are in the range 10^{33} – 10^{37} erg. These flare stars are, of course, in different parts of the evolutionary path from the sun but Lovell makes a strong case for the same flare mechanism being operative in M-, K- and main sequence stars.

It is possible, in principle, to set limits to the frequencies of large optical and cosmic-ray solar-type flares from other data: the cosmic-ray flux in ancient times inferred from analysis of lunar samples, deep-sea sediments, etc. and the frequency of magnitude changes (from the associated optical flares) in main sequence stars. The situation with ancient cosmic-ray fluxes seems to be that the average flux has remained constant within a factor of 2 over the past 10^9 yr and the flux over the past 400 yr is within 10% the same as that over the past $4 \cdot 10^5$ yr (ref. 15). Although the bursts only last for the order of days or hours their intensities would be so high (10^4 times 1 yr galactic flux at 3×10^9 erg cm⁻² for the linear extrapolation in Fig. 1) that they would contribute to long period averages. It is likely, therefore, that line *b* could continue to about 10^9 erg cm⁻² without producing conflict with the ancient cosmic-ray measurements but above that it seems necessary to have a somewhat more rapid fall off.

There is, however, one set of measurements which do not fit into this pattern. These are from the studies of ¹⁰Be and ²⁶Al in sea sediments^{16,17} which seem to show a 10-fold increase in cosmic-ray intensity for a period of $\sim 10^6$ yr some 2.5×10^6 yr ago. Such an increase could be due to a small number of large bursts spaced over the period and if true would correspond to an extrapolation of line *b* without reduction.

The situation with respect to magnitude changes of main sequence stars is not very clear. It has been pointed out that the biggest flares (in 1956–1960) seemed to have high conversion efficiencies to cosmic rays so that the optical flare energies were, like the cosmic-ray energies, $\sim 10^{33}$ erg. Presumably, for larger flares similar high efficiencies would result, so that, in order to achieve effects similar to SN at 10 pc, very infrequent optical flare energies of 'only' 10^{33} – 10^{34} erg would be required. Flaring to this extent would seem to produce negligible magnitude changes in a reasonable sample of nearby stars. Continuing to a flare energy of $\sim 3 \cdot 10^{38}$ erg, a factor of $\sim 10^6$ beyond the SN threshold, if the average large optical flare lasts ~ 1 d only, 10^5 stars (out of 1.5×10^{11} stars in the Galaxy) should change by ~ 1 mag for 1 d yr⁻¹. It seems likely that such a low frequency of flaring of this magnitude would not have been seen. Thus, the extrapolation of line *b* is allowable from astronomical data.

At this stage, it is obvious that the frequency of SN-equivalent cosmic-ray bursts is very high; some five orders of magnitude greater than calculated for SN at 10 pc. Thus, the effects postulated for SN would seem to occur with a very high frequency ($\approx$ every 1,000 yr). In fact, we feel that much bigger bursts are probably required in order to have a really large effect on the ozone layer in the upper atmosphere⁶ and a frequency of ~ 1 per 10^5 yr would seem more appropriate. Although this corresponds to a large extrapolation of line *b* there is a further large factor in hand because large bursts seem to be repetitive within the time constant (a few yr) for atmospheric ozone.

Reverting to supernovae, there is also the effect mentioned by Clark *et al.* of the increased cosmic-ray flux encountered when the solar system is inside a supernova remnant. The variation of cosmic ray intensity with time is the subject of conjecture; here we follow Clark *et al.* and adopt an increase over the local galactic intensity by 10^2 for the stage when the SNR radius is 10 pc. Assuming that the SNR merges into the I.S.M. when the radius is ~ 50 pc, the energy density over a 3-yr period (the approximate relaxation time of the atmosphere for O₃ production) follows the variation marked SNR (3 yr).

Allowance has been made for the lower efficiency of a galactic cosmic ray-type spectrum (of γ -rays) for depositing energy in the region of the atmosphere populated by O_3 (a factor of ~ 25 down in energy density).

Also indicated in Fig. 1 is the energy density integrated over the whole of the SNR (SNR p (all time)). This variation would be relevant to those biological processes involving gradual reduction in quality over many generations.

It is interesting to estimate the likely radiological dose rate at the Earth's surface from the big solar flares. The galactic flux generates ~ 30 mR yr $^{-1}$ at ground level (corresponding to $\sim 10^{-2}$ erg cm $^{-2}$ s $^{-1}$ of incident energy. Line b extrapolated indicates that a dose of 10^{11} erg cm $^{-2}$, corresponding to 10^4 R, would occur $\sim$ every 10^6 yr. Allowing for the eventual undoubted fall-off in $P(>\epsilon)$ below the extrapolated line such a dose (lethal even to very primitive forms of life) would probably still occur more often than once every 10^8 yr. Such large flux densities do not necessarily indicate flares larger by many orders of magnitude compared with those observed in 1959. More likely would be situations where flares just a few orders bigger occurred at a time when a favourable (?) magnetic field geometry focused a significant fraction of the output onto the Earth.

Finally, attention is directed to a quite different phenomenon: the possibility of extremely energetic bursts from the galactic centre. Briefly, there is the possibility that the 3 kpc 'ring' is due to a radially symmetric explosive event in the galactic nucleus. With the ejection of $\sim 2 \times 10^8 M_\odot$ the initial energy is at least 3×10^{68} erg (ref. 7). On this model, the explosion occurred 12–13 $\times 10^6$ yr ago (or earlier if there is oscillation of the ring). If the explosion resulted from the collapse of a very massive object then γ -ray emission would be very likely and, according to Colgate¹⁸, the fraction of energy in this form would be large. Extrapolating from the value for less massive objects we take $f \sim 10^{-3}$ giving $\sim 10^{65}$ erg in γ -rays. If $\sim 10\%$ of galaxies were to give bursts of this type then γ -ray bursts would occur at about the observed frequency.

With the γ -flash from the galactic centre the energy density at the Earth is $\sim 10^9$ erg cm $^{-2}$, a value that again exceeds the flux from local supernovae.

In conclusion, in addition to close supernovae, solar flares and explosions at the galactic centre should be borne in mind when considering possible catastrophes experienced by the Earth in its history. A more detailed study of solar and stellar flare data and ancient records of solar activity would appear to be profitable.

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Tunguska's comet and non-thermal ^{14}C production in the atmosphere

THE dramatic explosion on 30 June 1908 over Tunguska, Siberia continues to generate a wealth of literature on the supposed mysterious nature of the phenomenon. Hughes¹ recently summarised the generally convincing case for the hypothesis, first put forward by Whipple² and Astapovich³, that a comet collided with the Earth's atmosphere. Opposition to this theory and consequent support for less conventional hypotheses involving black holes⁴, almost critical masses of extraterrestrial fissionable material⁵, anti-matter bodies⁶ and alien spacecraft⁷ have been based substantially on the non-observation of the comet before impact, the explanation of height of the explosion above the Earth's surface, the composition of the glassy spherules found at Tunguska and finally, and of most importance, the apparent occurrence of nuclear phenomena in the explosion as indicated by the subsequent enhancement of radiocarbon in the atmosphere. In the following, we discuss the first and last of these points which are clearly the most important.

Ben-Menahem⁸ re-analysed the old seismograms of the Tunguska event and deduced the parameters of the original explosion by comparing the old data with contemporary records of the seismic and acoustic effects of recent nuclear air explosions. He concluded that the main explosion had an energy of $(5 \pm 1) \times 10^{23}$ erg and occurred at a height of 8.5 km. The size of the incident body can be estimated by assuming that its original kinetic energy was entirely converted into explosive energy. Krinov⁹ found the geocentric velocity to be between 28 and 50 km s $^{-1}$, Levin¹⁰ gave a geocentric velocity of 47 km s $^{-1}$. This latter value gives the body a mass of 5×10 g. Assuming the body is a cometary nucleus, of density 1.14 g cm $^{-3}$ (ref. 11) its equivalent diameter is found to be 40 m. The absolute visual magnitude, m_0 of a comet of mass M (its magnitude 1 AU from both the Sun and Earth) is given by¹²

$$\log_{10}(M \text{ in g}) \approx 21 - 0.4 m_0 \quad (1)$$

so the Tunguska comet had $m_0 = +26$.

The apparent magnitude, m of the comet when it is at a distance r AU from the Sun and Δ AU from the Earth is given by¹²

$$m = m_0 + 5 \log_{10} \Delta + 10.5 \log_{10} r \quad (2)$$

The two equations given above are mean, rule of thumb relationships obtained by considering the observed parameters of a great many comets. Some comets are notorious for departing from expected behaviour but in the absence of any observations of the Tunguska comet we can only assume that it was one of the more well behaved members of its species and obeyed the equations given.

Table 1 Apparent magnitude, m and time to impact t of the comet as a function of its distance from the Earth

Δ (AU)	r (AU)	m	t (min)
0.002	1.0	12.4	104
0.001	1.0	10.9	51
0.0005	1.0	9.4	24
0.0002	1.0	7.4	8
0.0001	1.0	5.9	3

Values of m for certain specific values of Δ are given in Table 1. Also given in Table 1 is the time t taken by the comet to travel, at 47 km s $^{-1}$, from a point Δ AU away from the Earth to the Earth's surface. At the turn of the century comets were usually discovered at magnitudes around +11. The Tunguska comet is only brighter than this value for about 1 h before it impacts with the Earth, ample reason for it not being seen on its earthward path. Fesenkov¹³ finds that, for all possible cometary orbits calculated from the fireball trajectory, the angular distance between the comet and the Sun is small. When the comet encountered the Earth it would be coming from a point in the dawn sky comparatively

close to the Sun and would thus be most difficult to detect and observe. The orbital configuration is similar to Comet Mrkos which was only detected after having rounded the Sun and travelled beyond the Earth's orbit.

It is most difficult to calculate the expected flux of these very small cometary nuclei to the Earth's surface because of the lack of relevant observational data. Vsekhv'yatskii¹⁴ gives cumulative number data for comets with $1 < \text{mag}_0 < 14$. A wanton extrapolation of Whipple's¹⁵ interpretation of this data gives a value of 7×10^8 for the number of comets brighter than $\text{mag}_0 = +26$ which pass within 1.2 AU of the Sun within 100 yr. Using Everhart's¹⁶ calculations the chance of such a comet striking the Earth is about 5×10^{-2} in 100 yr; so one on average should hit the Earth every 2,000 yr.

Comparing the ablation and deceleration of the cometary nucleus with that of a meteoroid forming a meteor train it can crudely be assumed that one atom (or molecule) of the incident body is ablated (albeit with a high energy) when it is struck by an atom (or molecule) of the atmosphere. The cometary nucleus thus explodes when it has collided with a mass of air equal to its own mass. Assume that the atmosphere has a density, ρ , at a height h of $\rho = \rho_0 \exp(-h/H)$ where ρ_0 is the density at ground level and H is the scale height (~ 7 km). The cometary nucleus (radius r_c , density ρ_c moving on a trajectory inclined at an angle χ to the vertical will intersect a mass M_c in descending to height h_1 , where

$$M_a = \pi r_c^2 \sec \chi \rho_0 \int_{h_1}^{\infty} \exp(-h/H) dh \quad (3)$$

M_a is equal to the comet mass ($4\pi\rho_c r_c^3/3$) at the explosion height h given by

$$h_c = H \log_e \left(\frac{3 \sec \chi \rho_0 H}{4\pi r_c \rho_c} \right) \quad (4)$$

Putting $h_c = 8.5$ km, $H = 7.1$ km, $\rho_0 = 10^{-3}$ g cm⁻³, $r_c = 2.1 \times 10^3$ cm and $\rho_c = 1.14$ g cm⁻³ gives $\chi = 1.5^\circ$. Fesenkov¹³ concluded that χ was between 65° and 70° and probably larger, Levin¹⁰ calculated χ to be 82° . The low value calculated above is probably due to the initial assumption of a one to one ratio between collision atoms and ablated atoms. The formation of an aircap around the nucleus in the lower, denser, regions of the atmosphere will lessen the ablation and enable it to traverse a longer atmospheric path and thus have a higher value of χ .

Magnetite and silicate globules, 80–100 μm in size, were recovered from the soil near the explosion site. Detailed analysis of these revealed small amounts of Co and Ni as well as traces of Cu and Ge¹⁷ and this has been offered as partial proof of the 'alien spacecraft' hypothesis. It must be remembered, however, that these elements are most probably present in the dust of the cometary nucleus at their normal cosmic abundances. Their high values of ionisation potential vitiate against them showing up in cometary spectra.

One of the major stumbling blocks of the cometary impact Hypothesis is the ¹⁴C anomaly that is said to have occurred in 1909. Cowan *et al.*⁶ found that the ¹⁴C activity was higher in that year than normal and deviated from the mean by over two standard deviations (but see ref. 18). Cowan *et al.*⁶ manufactured this extra radiocarbon in the atmosphere by proposing that 1/7 of the Tunguska energy was due to anti-matter annihilation. Hunt *et al.*⁵ produced this ¹⁴C excess by a nuclear explosion at Tunguska. A sudden influx of ¹⁴C into the Earth's atmosphere will be observed as a positive anomaly in the graph of radioactive CO₂ content as a function of time, this anomaly having a width (to half maximum values) of about 2.4 yr. This is because atmospheric CO₂ is continually being removed by plants in the biosphere, converted to humus and then returned to the atmosphere as this humus decays; 5% is absorbed each year in this way. CO₂ also dissolves into the oceans, rainfall helping considerably, and this removes about 20% yr⁻¹ (see ref. 19). So 25% of the atmospheric CO₂ is being locked away each year, remains locked away for between 10 and 50 yr and

is subsequently slowly returned by plant decay and oceanic evaporation.

Now, if all the kinetic energy of the comet (5×10^{23} erg) were converted into heating its constituent particles and an equal mass of the atmosphere (mean mass 12) the temperature obtained would be about 2×10^6 K. Since this is substantially sub-nuclear it has been argued that normal cometary impact could not have produced the observed radiocarbon. It is entirely fallacious to suppose that sub-nuclear temperatures cannot produce nuclear effects as can be seen by consideration of the solar flare phenomenon. In a flare, the mean temperature of the particles is certainly less than 2×10^7 K, that is an energy of order 10^{32} erg distributed among some 10^{16} g. Even at the low densities of the solar atmosphere, many associated high energy phenomena are seen to occur—the production of X and γ rays, MeV electrons and GeV nuclei²⁰. The mechanism by which these are produced is still not properly understood but qualitatively may be attributed to stochastic acceleration of particles in the turbulent spectrum of plasma waves generated in the flaring atmospheres by the primary energy release. Although this process is magnetohydrodynamic in character the solar magnetic field need not play any direct part (other than as the source of energy) since the large particle velocities and the ionisation produced by any explosion can generate the necessary magnetohydrodynamic conditions. Similar results are, therefore, to be expected in the Earth's atmosphere following a cometary impact.

We can estimate the ¹⁴C production in the Tunguska cometary impact by a rough scaling comparison with the flare results, without specifying the detailed process. The collisional production of energetic neutrons by the accelerated particles varies as $nNQV\tau$ where n is the atmosphere number density in the heated atmosphere, N the total number of particles in the heated volume, Q the neutron production cross section, V , the mean particle velocity, and τ the duration of the event. Thus if v is the number of neutrons produced and the comet and flare characteristics are denoted by subscripts C and F then

$$v_c = v_f \frac{n_c N_c Q_c V_c \tau_c}{n_f N_f Q_f V_f \tau_f} \quad (5)$$

From the cometary mass of 5×10^{10} g we have $N_c \approx 2 \times 10^{33}$ while the atmospheric density at 8.5 km gives $n_c \approx 2 \times 10^{19}$ cm⁻³ and for a flare $N_f \leq 10^{40}$. The density in the flare acceleration region is uncertain but is probably around $n_f \approx 10^9$ cm⁻³, and the typical flare duration is $\tau_f = 100$ s. Chupp *et al.*²¹ have set an observational upper limit to the number of 20 MeV neutrons formed even in a 2B flare as $v_f \leq 10^{25}$. Thus from equation (5) we have

$$v_c \approx 3 \times 10^{26} \left(\frac{Q_f V_c}{Q_c V_f} \right) \tau_0 \quad (6)$$

The factor in parentheses in equation (6) is uncertain because the detailed process of neutron formation is unknown. Certainly the typical nuclear collision speed V_c in a cometary impact explosion may be less than that in a flare (V_f) since the explosion temperature driving the plasma process is lower. On the other hand, the neutrons proposed by Cowan *et al.*⁶ for radiocarbon formation are only of about 10 MeV energy so Q_c/Q_f may be substantially larger than unity. If, therefore, we estimate the whole parenthetical term in equation (6) as unity then we expect 3×10^{26} τ_c neutrons to be formed in a Tunguska-type cometary impact. By comparison Cowan *et al.*⁶ estimate that about 10^{27} neutrons are required to explain the radiocarbon data. Thus, if the necessary hot plasma conditions were sustained in the Earth's atmosphere for at least 3 s (a very plausible figure compared with the duration of enduring meteor trails, overdense diffusion times, etc.) then the required neutron formation would probably ensue by non-thermal plasma processes.

We conclude, therefore, that within the uncertainties of the effect of chemical composition on collective plasma acceleration processes and on collisional neutron production, the number of neutrons expected in the Tunguska impact, as scaled from solar

flares, is in remarkably good agreement with the radiocarbon data requirements. Also the low mass of the comet (5×10^{10} g) coupled with its extreme faintness ($\text{mag}_0 = +26$), and its position in the dawn sky, just before impact makes its prior discovery extremely unlikely. These points strongly support the suggestion that the Tunguska explosion was caused by an impacting small comet and that nothing more exotic need be invoked.

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Antarctic topography from balloons

ANTARCTIC ice surface elevation maps are far from being complete, due to the difficulties in applying regular surveying and mapping techniques in Antarctica. A new wealth of ice sheet surface topography data is now available, from many balloons, floating at an altitude of about 12.5 km, which traversed the Antarctic during its 1975 summer. These balloons were part of 411 constant density balloons launched in the Southern Hemisphere during the Tropical Wind, Energy conversion and Reference Level Experiment (TWERLE)¹. Each balloon carries three sensors: radio altimeter², pressure sensor³ and ambient temperature sensor. The balloon transmits data once per minute, as long as its solar panel is sufficiently illuminated⁴. The data are received by the Random Access Measuring System (RAMS)⁵ on board the NIMBUS-6 satellite, whenever the balloon is within line of sight.

The near Polar orbit of NIMBUS-6, and the continuous summer daylight combined to allow continuous coverage of TWERLE balloons, south of 66°S, during December 1975 and January 1976. Continuous coverage means 13–14 passes per balloon in 24 h. The longest pass is an overhead one, which lasts more than 15 min, hence the data consists of 15 transmissions, or less, per pass. In addition to recording the balloon's sensors data, RAMS also measures the received carrier frequency. The Doppler history of up to 15 transmissions during an overpass yields the balloon position⁶. Two consecutive passes can produce the wind information.

The average balloon life during TWERLE was slightly more than two months, with some balloons lasting more than a year. The launching strategy and the general circulation at balloon height caused a typical daily concentration of about 40 balloons south of 45°S, during December 1975–January 1976. Many of these balloons traversed the Antarctic Continent.

The extent of Antarctic coverage by the TWERLE balloons is summarised in Fig. 1. This gives the total coverage from all the balloons during the period 1 November 1975–19 March 1976. Each symbol on the map indicates the total number of measure-



Fig. 1 TWERLE balloons coverage over Antarctica 1 November 1975–19 March 1976. Each symbol indicates the number of measurements over a square 55×55 km ($1 = 1, \dots, 10$, $A = 11, B = 12, \dots$). The data boundary corresponds to 65°S, and 0° longitude is up.

ments over a square of 55×55 km. A measurement is the average of up to 15 balloon transmissions, 1 min apart, recorded during the satellite overpass. As Fig. 1 shows there is almost complete coverage over Antarctica, with many squares being traversed more than once.

The radio altimeter data over Antarctica resembles over-water data, as far as standard deviation of the 15 altitude readings per pass. This small standard deviation is due to the flatness of the ice cover. The ice flatness further contributes to the measurement accuracy by reducing the adverse importance of position error, which is specified at 5 km.

To obtain the surface elevation from the altimeter readings it is necessary to know the balloon's altitude above sea level (a.s.l.). Constant altitude could not be assumed because (1) the altitude of a constant density level is not constant, and (2) the balloon equilibrium density changes following diurnal cycles. What can be assumed constant, for several hours at least, is the altitude a.s.l. of a pressure level along the balloon trajectory. This is the result of the quasi-geostrophic nature of the wind. In other words, the balloon, which follows the wind, is moving parallel to the isobars. It can be concluded that the balloon altitude a.s.l. could be calculated if the altitude of a reference pressure surface above the balloon subtrack is known once a day. 150 mbar was chosen as this reference pressure, because it is the standard radiosonde level nearest to the balloon flight level.

The relation between the surface elevation, the altitude of the 150 mbar pressure surface and the altimeter reading is

$$E = Z(150 \text{ mbar}) - (P + P_{\text{corr}} - 150) (0.2T + 53.7) + (75 \cos^2 \Phi - 15) - h \quad (1)$$

where E is the surface elevation a.s.l. in metres, $Z(150 \text{ mbar})$ is the altitude of the 150 mbar surface in geopotential metres, P is the pressure sensor reading in mbar, P_{corr} is the pressure sensor correction in mbar, T is the ambient temperature reading in °C, Φ is the latitude and h is the altimeter reading in metres.

The second term on the right hand side is the altitude difference between the balloon's pressure level and the 150-mbar pressure level, using a linear approximation to the hydrostatic

relation. The third term is an approximation to the difference in the balloon altitudes when given in units of geopotential metres and geometric metres. P_{corr} is the difference between true pressure and the pressure sensor reading. Once in altitude, the TWERLE pressure sensor is very stable. Erroneous pre-launch calibration, however, and very long term drift may cause a bias error of up to 3 mbar. This bias error can be removed by several comparisons with 150-mbar synoptic maps, when the balloon flies over water in an area well covered by radiosonde data, such as Australia and New Zealand.

The altitude of the 150-mbar level above the balloon subtrack, for use in equation 1, should also come from radiosonde data. The radiosonde network in Antarctica is, however, too sparse to produce good synoptic maps. To our help come all the other TWERLE balloons in the area. Balloons over water provide both the geopotential height of the pressure surface and the wind. Balloons over land are used for wind only. Figure 2 is one of the synoptic maps drawn for the analysis of a test case balloon no. 766. On that day, there were only nine radiosonde stations south of 45°S which reported the wind and/or the geopotential height of the 150-mbar level, at the mandatory time of 1200 GMT. There were, however, 32 more TWERLE balloons south of 45°S reporting the same information.

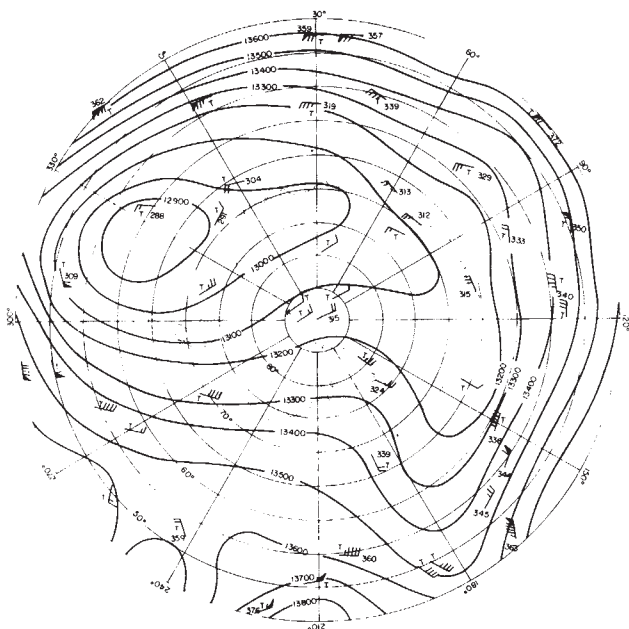


Fig. 2 Synoptic map of the 150 mbar pressure surface at 1200 GMT, 11 December 1975. Constructed from nine radiosonde reports and 32 TWERLE balloons (marked with 'T'). The balloon marked 'X' is no. 766, whose full trajectory appears in Fig. 3.

The first balloon to be analysed during its Antarctic traverse was balloon no. 766. Its trajectory from 3 December 1975 till 14 December 1975 is plotted in Fig. 3. Next to each position, the elevation of the Antarctic continent calculated using Equation 1 is given. P_{corr} for balloon no. 766 was checked six times during the 100 d preceding the traverse, and was found to be 1.7 mbar. Six synoptic maps, similar to Fig. 2, were plotted for the traverse period.

The estimated error budget in the calculated elevation is as follows: pressure surface: ± 30 m, altimeter: ± 10 m, pressure sensor: ± 10 m, position error of 5 km at a slope of 2 m km^{-1} : ± 10 m. Adding all these yields a worst-case error of ± 60 m. This accuracy is comparable to accuracies of ± 30 m obtained during aircraft radio echo soundings⁷, and to accuracies of ± 50 m obtained in over-snow traverses using the two barometric altimeters method⁸.

There are several absolute check points available for the elevation data in Fig. 3. First there are three measurements of the sea surface elevation before crossing the shore line at the

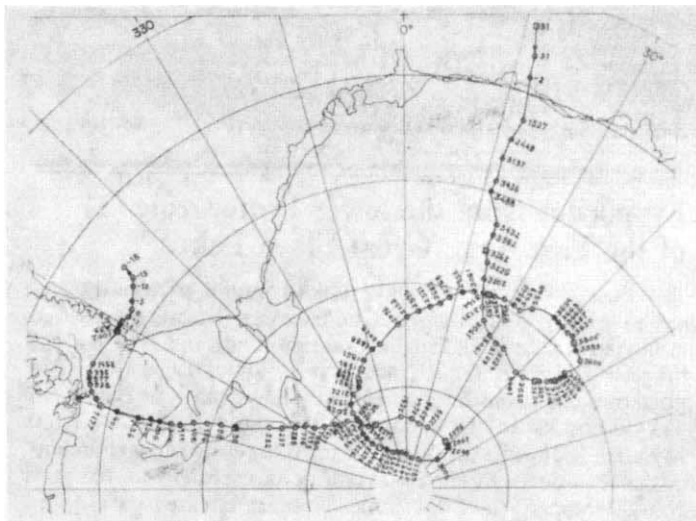


Fig. 3 The traverse of TWERLE balloon no. 766 over Antarctica, 3-14 December 1975. The Antarctic surface elevation at the balloon subtrack was calculated using equation (1), and is given in metres. The first known position on a GMT day is marked by a square.

beginning of the traverse. These exhibit a maximum error of 31 m. Then there are the sea surface measurements at the end of the traverse, which exhibit maximum error of 18 m. A relative check is available at the poleward most crossover in the trajectory. On 8 December 1975 an elevation of 2,038 m was obtained when the balloon passed over position 85° 97'S, 324° 25'E. Three days later, an elevation of 2,042 m was obtained for 85° 98'S, 324° 29'E.

Another indication of the accuracy is available from a preliminary comparison with the Radio Echo Sounding Map of East Antarctica⁷, performed on 33 well distributed localities, measured from five different balloons. The average error was +7 m (balloon data being higher), and the standard deviation of the differences was 36 m. Whenever the balloon subpoint did not coincide with an elevation contour line of the radio echo map, a linear interpolation between the two neighbouring contour lines was used. In five out of the 33 comparisons, the differences were larger than 60 m (the largest being -83 m), however, it should be noted that the sum of the two accuracies is ± 90 m. It can be concluded that the accuracy of the data, and the extensive coverage promise significant additions to the Antarctic ice maps. (The data already analysed is available from Nadav Levanon, in tabulated form.)

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Evaporites from the lower Proterozoic of the East Arm, Great Slave Lake

THE East Arm of Great Slave Lake contains excellently preserved lower Proterozoic sedimentary rocks that were deposited in fluvial, coastal and shallow marine conditions. These rocks range in age from about 2,400 Myr to about 1,600 Myr. The polar wander curve for this period¹ shows that the East Arm was within 30° of the equator during the period 1,900–1,700 Myr. The sediments deposited during this period show abundant evidence of deposition in arid conditions, especially in that they contain indications of the former presence of extensive evaporites. Evidence of former evaporites is lacking in those sequences deposited apparently beyond 30° from the palaeoequator. Permian to modern marine evaporites are generally confined to $\pm 30^\circ$ of latitude. It is proposed, therefore, that climatic (and by possibly atmospheric and hydrospheric) conditions were grossly similar in the lower Proterozoic to those of today. These deposits pre-date similar sequences in the MacArthur River area by some 300 Myr (ref. 2). They predate the supposed initiation of sulphate evaporites by 1,000 Myr (ref. 3) and clearly negate Cloud's various hypotheses about the evolution of the Earth's atmosphere and hydrosphere.

The East Arm of Great Slave Lake is a graben within which there was intermittent sedimentation and igneous activity from 2,500 Myr ago to 1,300 Myr ago⁴. Three major groups have been defined⁵. (1) The Wilson Island Group. A group of shallow water sedimentary rocks and volcanics older than 2,200 Myr;

(2) The Great Slave Supergroup, deposited between about 2,200 Myr and 1,790 Myr; (3) The Et-Then Group, deposited between ~1,700 Myr and 1,600 Myr.

The Wilson Island Group has been metamorphosed and deformed, although locally there are well preserved, unmetamorphosed sections. The other groups are unmetamorphosed and beautifully preserved. Throughout much of the lower Proterozoic deposition was in shallow water and fluvial, tidal-flat and shallow marine sediments are abundant (Table 1).

The polar wander path for North America¹ indicates that the East Arm within 30° of the equator during the deposition of the middle and late Great Slave Supergroup (from about 1,900 Myr to about 1,700 Myr). The bulk of Permian-modern marine evaporites has been essentially confined to $\pm 30^\circ$ of latitude⁶. The depositional environments in the East Arm indicate that ideal conditions for evaporite formation were frequent. The palaeomagnetic data indicate that we could expect evaporites in the sequence between 1,900 Myr ago and 1,700 Myr ago. In Table 1, we show those formations that contain evidence for evaporites and we document the evidence below.

The oldest unit in which there is evidence for evaporites is the Kluziai Formation. In the Snowdrift area, there are nodules of slightly ferroan dolomite within fine-grained, well-sorted sandstones. The nodules are usually restricted to certain horizons. They are 1–2.5 cm in diameter and usually consist of a single poikilotopic dolomite crystal. Similar nodules occur in the top of the Hornby Channel Formation in the same area but are not well preserved. The nodules are similar to ones found in the Zambian Copperbelt sandstones, and Annels⁷ has shown how carbonate has replaced plates of anhydrite and gypsum which are still preserved in places in these rocks. It is proposed that the nodules in the Kluziai Formation have replaced original anhydrite.

Additional evidence for evaporites in the Sosan Group comes from the Akaitcho River Formation on Seton Island where

Table 1 Evidence for the presence of evaporites in the East Arm, Great Slave Lake

	Group	Formation	Lithology	Deposition environment	Evidence for evaporites
	1200				
	Et-Then	Preble Murky	Sandstones Conglomerates	Fluvial Alluvial Fan	
	1795				
	Christie Bay	Diorite Intrusion Pearson Portage/Inlet Tochatwi Stark	Basalts and argillites Red shale, ssts and slts Sandstones Dolomites and Red Mdsts	Alluvial Plain? Sabkha? Marine to fluvial Tidal arid coast	Halite and gypsum casts Casts of anhydrite nodules? Halite casts and broken beds
	Pethei	Hearne Wildbread Pekanatui Point Blanchet McLean Utsingi Taltheilei Douglas Peninsula	Limestones Limestones Limestones and shales Turbiditic sandstones Red/brn mdsts and Lmsts Limestones Dolomites Red siltstone and mdst	Shallow marine Shallow marine 'Deep' marine 'Deep' marine 'Deep' marine Supratidal Shallow marine Sabkha	Broken beds, crinkled laminae Gypsum casts, broken beds
	Kahochella	Charlton Bay McLeod Bay Gibraltar Seton	Green shales with concretions Red shales with concretions Red shales Alkali volcanics	Shallow marine Intertidal Supratidal, arid coast	Gypsum casts Gypsum casts Gypsum casts
	1872				
	Sosan	Akaitcho River Kluziai	Red sandstones and slts Sandstones	Coastal, Deltaic Fluvial/marine	Gypsum casts Dolomite nodules after anhydrite?
		Duhamel Hornby Channel	Dolomites Pebble sandstones	Shallow marine Fluvial to shallow marine	Dolomite nodules after anhydrite
	2200				
	Union Island		Basic volcanics, Dolomites and Red and black shales	Shallow marine	
	Wilson Island		Volcanics, Quartzites, Dolomites and siltstones		
	> 2485	Basement			

Great Slave Supergroup

Olade and Morton⁸ described pseudomorphs after gypsum from red siltstones and shales. These sediments are overlain by volcanics of the Seton Formation, which have been dated on Seton Island at 1,872 Myr (ref. 9). In the Akaitcho River Formation at Reliance, there are small (0.5 mm) nodules of length slow chalcedony (quartzine), which may indicate replaced evaporites¹⁰. It is interesting that these occur in well-sorted glauconitic sandstones. The glauconite occurs as pellets which have cracks infilled with carbonate—a feature that has been ascribed to desiccation¹¹. In these two areas, the Formation was deposited in shallow marine, perhaps intertidal conditions. On the north shore, at Talthelei Narrows, red mudstones of the Akaitcho River Formation contain beds with haematite pellets and carbonate nodules as well as casts after gypsum. These beds are interpreted as pedogenic horizons and probably were formed in a sabkha environment.

Pseudomorphs after gypsum are also common in the Kaho-chella Group. They occur in the upper parts of the Gibralter Formation in intertidal red mudstones with stromatolites, intraformational conglomerates and pelletoid ironstones¹². Casts after gypsum have been described from tufts of the Seton Formation, again associated with pedogenic horizons, and from red shales with carbonate concretions in the McLeod Formation near Reliance. The Douglas Peninsula Formation contains similar casts in red fissile mudstones with carbonate or haematite/jasper laminae. Hoffman¹³ has tentatively proposed a lacustrine origin for these rocks which probably formed in a sabkha environment. During the remaining Pethei Group times shallow-water sediments were only deposited on a northern platform. Of the platform sequences, the Utsingi Formation of stromatolitic limestones and dolomites has been tentatively described as a supratidal sabkha deposit¹². We have observed evaporite breccia and contorted laminae within the unit at Reliance. Both features resemble closely those in the Purbeck Formation of Dorset, where the latter has been cited as part of the evidence for the former presence of evaporites¹³.

On Isui Island, there is a sequence of fissile, red, calcareous mudstones containing thin laminae of red-stained carbonate and calcite pseudomorphs after anhydrite. The sequence is in fault contact with other units, but closely resembles the Douglas Peninsula Formation with which it is tentatively correlated¹⁷. The laminae are disturbed by small scale cross-lamination and scours, but also by crinkle folds (1 cm amplitude) which probably formed while the rock was still plastic. There are plugs of brecciated, banded drusy calcite up to 3 m across cutting these sediments. The plugs contain clasts of red dolomitic mudstone. Plugs of oligomictic breccia with no calcite cement are also seen to cross-cut bedding. The drusy calcite contains streaky layers of red calcareous mudstone. Rosettes and euhedral crystals of barite occur in bands in these layers of calcareous mudstone and overgrow the drusy calcite. These features closely resemble the upper zone of the cap rock of a salt dome¹⁴ except that they are on a far smaller scale. They are, however, also similar to structures produced by small scale movements over anhydrite beds¹⁵. There is no evidence for halite in nearby rocks, but pseudomorphs after anhydrite are abundant. We, therefore, suggest that these plugs were perturbations in the top of an anhydrite bed that has subsequently been calcitised.

In Christie Bay Group times, the polarity of platform and basin broke down, and once again shallow marine to arid coastal conditions prevailed over much of the East Arm. The best evidence of former evaporites comes from the Stark Formation which consists of dolomites, red mudstones and siltstones. Stromatolite mounds, crinkled algal laminae, flat pebble conglomerates, wave ripple marks, and loading and shrinkage cracks all point to an intertidal to supratidal origin^{12,16}. The laminated dolomites are often highly brecciated, and there are various types of breccia. In places, almost the entire thickness of the formation (in excess of 1,000 feet) is disrupted to a megabreccia. Elsewhere small patches of breccia occur amidst undisturbed strata. The breccias were attributed to gravity tectonics around rising diorite laccoliths,^{4,12} an hypothesis

rejected by Badham¹⁸ who proposed that they were analogous to evaporite breccias elsewhere and the result of collapse after solution of evaporites. More recently Hoffman *et al.*¹⁹ have concurred with this view and have proposed an elegant model.

The Stark Formation retains evidence, other than the breccias, for the former presence of evaporites. The dolomites contain vugs wholly or partially filled with ferroan dolomite and younger calcite. The vugs are up to 2.5 cm long and compaction has occurred around them. They are confined to pyritic, stromatolitic dolomite and are most common in the base of algal mounds. They disrupt original laminae and are associated with shrinkage cracks. The association is similar to that in the Middle Jurassic of Scotland³. The vugs are similar to the elongate fenestrae of the medium laminoid fenestral fabric and are assumed to have once contained gypsum²¹. The red mudstones contain abundant and well developed pseudomorphs after halite. These are now usually filled with dolomite, but in the aureoles of the diorite laccoliths feldspar is the replacing mineral. It is suggested that the Stark formation originally contained thick sequences of evaporites, most of which were removed in solution with concomitant collapse of overlying strata. Some of the breccias and deformation within the Upper Christie Bay Group may, however, be due to salt diapirism—a hypothesis which needs more detailed investigation.

The youngest evaporites in the East Arm occur in the Portage Inlet Formation. Pseudomorphs after halite are common in buff sandstones and shales, which commonly also contain desiccation cracks. Mudstones at the top of the Formation contain pseudomorphs after rosettes and single crystals of gypsum¹².

There is, therefore, abundant evidence for evaporites in the middle and late Great Slave Supergroup. Much, but by no means all, of this evidence is from pseudomorphs after gypsum. Gypsum may also form during the degradation of sulphides²². The nature of the sediments and the other evidence of evaporites suggest, however, that the gypsum was of evaporitic origin. Many of the East Arm evaporites were deposited on supratidal flats and sabkhas, an environment where similar sediments with evaporites are forming today. Evidence for former evaporites is found only in the middle and late Great Slave Supergroup and not in the Union Island and Lower Sossan Groups, when equally suitable depositional environments existed. This suggests that (1) the distribution of lower Proterozoic climatic belts (and thence the geographic distribution of evaporites) was broadly similar to that of today and (2) the palaeomagnetic data are correct.

Finally it is interesting to note evidence for the former presence of evaporites from rocks of the Belcher Group (in Hudson Bay) of similar age to the Upper Great Slave Supergroup²³. This evidence supports the above conclusions.

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Polycyclic aromatic hydrocarbons in long-range transported aerosols

THE presence in Norway of long-range transported air pollutants from the European continent and England is well established. In particular, SO_2 and other inorganic compounds in precipitation have been studied¹. Less attention has been paid to the organic constituents of the long-range transported pollutants. Several organic components have been identified in precipitation in Norway², including alkanes, polycyclic aromatic hydrocarbons (PAH), phthalic acid esters, fatty acid esters, fatty acids, and dicarboxylic acids. In addition some chemicals typically connected to industry and industrial products are present. Long-range transfer of PAH compounds, in particular, is of environmental interest since a number of such compounds cause cancer in animals and they are also suspected to be human carcinogens³. We report here preliminary results on the PAH-content of long-range transported and stationary air masses.

All samples were taken by the Norwegian Institute for Air Research at their sampling station at Birkenes in southern Norway. Airborne aerosols were collected by high-volume samplers on glassfibre filters. About 2,500 m³ air was drawn through the filters per day, and the filters were changed every 24 h. The origin of the air masses transferring the pollutants has been indicated by trajectories which

are calculated on the basis of meteorological data⁴. When the trajectories showed that consecutive filters were sampling air of the same origin, the filters were combined before analysis.

The air filters reported in this work represent two sets. The first set contains samples of air which had come from France/England (sample 1) and England/Scotland (sample 2). The other set contains samples of air coming from northern Norway (sample 3) or of stationary air from southern Norway (sample 4). The samples represent air sampled for 24, 48 and 72 h.

The analytical procedure used in this work is based on the method of Grimmer and Böhneke⁵. Gas chromatographic analysis was performed using a Carlo Erba Fractovap 2101 A gas chromatograph equipped with a 50 m × 0.34 mm i.d. glass capillary column coated with OV 1. The technique of splitless injection of the sample was used⁶. Retention times and peak areas were registered automatically by means of an electronic integrator. The PAH compounds were identified by comparing the chromatograms with those of the PAH-standards and the amounts were determined comparing the electronically integrated areas of each peak with those of the internal standards.

Results of the PAH analyses are given in Table 1 and a typical gas chromatogram is shown in Fig. 1. It is seen from the chromatogram that the system used is capable of separating critical isomers such as benzo(a)pyrene from benzo(e)pyrene and benzo(a)anthracene from chrysene and triphenylene. This separation is essential for characterising the potentially hazardous PAH components in environmental samples.

Table 1 shows that 20 different PAH compounds have been identified and determined. These include some of those reported to be carcinogenic³. It is also shown that the samples from western Europe contain about 20 times more PAH than the samples from northern Norway and the stationary air from southern Norway. Although these samples are taken over short periods only, and are not neces-

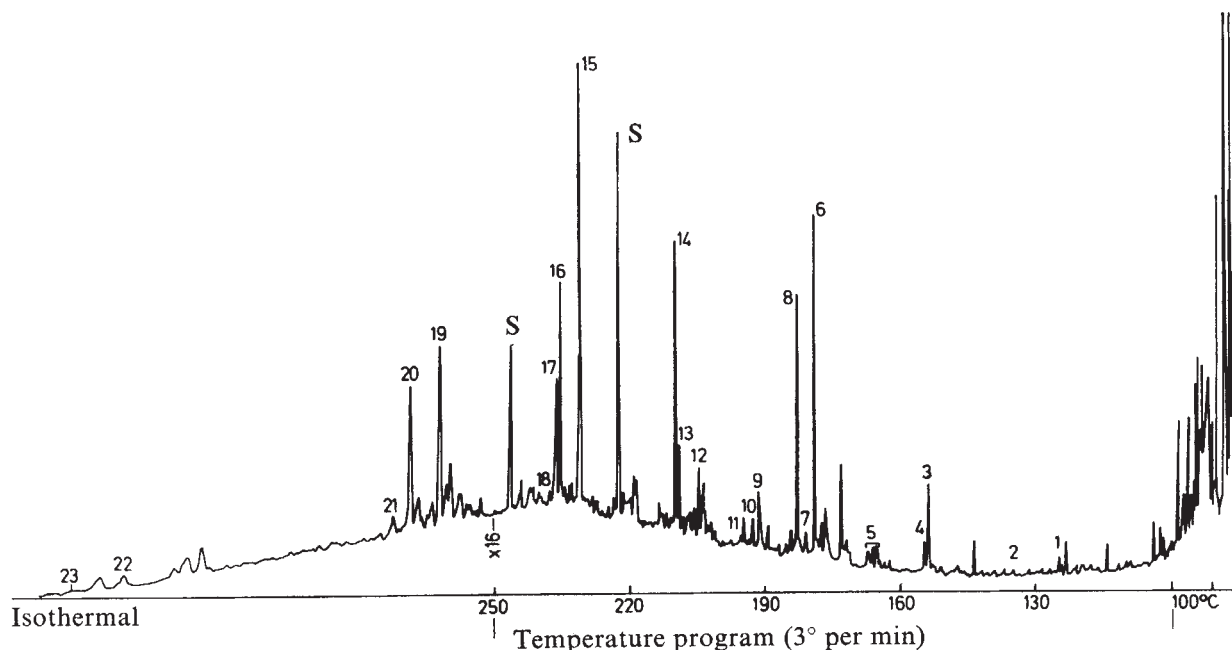


Fig. 1 Gas chromatogram of air filter sample originating from northern England and Scotland. The combined air filters were extracted with 50 ml cyclohexane for 16 h in a Soxhlet apparatus. Before extraction two internal standards, β/β' -binaphthyl and *m*-quaterphenyl, were added. The solution was then extracted with 50 ml dimethylformamide (DMF)/water(9:1). The extraction was repeated with 25 ml of the DMF-water solution. 100 ml water were added to the combined DMF-water solutions and subsequently extracted twice with a total of 75 ml cyclohexane. The cyclohexane-solution was washed twice with distilled water, dried with Na_2SO_4 , and concentrated to about 10 ml on a modified Kuderna-Danish apparatus under reduced pressure. The extract was further concentrated to about 0.5 ml on a heatblock (30 °C) under a gentle stream of highly purified nitrogen. The peak numbers refer to the numbers in Table 1. The main components are (3), phenanthrene; (4), anthracene; (6), fluoranthene; (8), pyrene; (15), benzo (*b&k*) fluoranthene; (16), benzo(*e*) pyrene; (17) benzo(*a*)pyrene; (18), *o*-phenylenepylene; and (19), benzo(*ghi*)perylene. S, Internal standards.

Table 1 Concentration of PAH in aerosols (ng per 1,000 m³)

Sample number Sampling period	1	2	3	4
	20–21 February 1976	25–26 November 1975	25–27 January 1976	1–4 February 1976
PAH	Origin of air:			Stationary air, Southern Norway
	England, France	Northern England, Scotland	Northern Norway	
3 Phenanthrene	} 4,725	1,216	36	146
4 Anthracene		278	68	—
5 Methyl-phenanthrene/-anthracene	661	216	—	52
6 Fluoranthene	6,637	3,965	171	324
7 Dihydrobenzo (a & b) fluorenes	874	363	32	32
8 Pyrene	4,864	3,293	135	286
9 Benzo (a) fluorene	815	318	21	26
10 Benzo (b) fluorene	571	149	117	148
11 1Methylpyrene	147	99	9	9
12 Benzo (c) phenanthrene	1,021	957	38	108
13 Benz (a) anthracene	585	740	41	73
14 Chrysene/Triphenylene	1,756	3,269	99	194
15 Benzo (b & k) fluoranthene	4,312	4,013	83	467
16 Benzo (e) pyrene	1,191	2,635	66	135
17 Benzo (a) pyrene	965	2,053	59	98
18 Perylene	90	191	Trace	11
19 o-Phenylene-pyrene	1,306	1,920	62	144
20 Benzo (ghi) perylene	1,142	1,971	64	140
21 Anthanthrene	225	423	7	22
22 Coronene	212	183	—	20
Total identified PAH	32,099	28,252	1,108	2,435

sarily representative of air that has travelled from England, the concentrations of benzo(a)pyrene and some other PAH's are of the same order of magnitude as the mean values reported in recent years in London⁷. The results of this study, though preliminary, show clearly the presence of PAH in atmospheric long-range transported aerosols. Studies have now been extended to incorporate further samples and other sampling stations.

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The microbe thermistor

WE describe here the development of a new analytical configuration, called a microbe thermistor, in which immobilised micro-organisms are placed in proximity to a thermistor which measures the metabolic heat evolved by them. Many metabolites can thus be analysed, as we show here by the determination of both single components such as glucose and fructose and of more complex mixtures such as casein. This is in contrast to other analytical applications of immobilised enzymes, for example enzyme electrodes^{1–3} and enzyme thermistors^{4–6}, which can normally allow analysis of only one compound at a time.

We used immobilised whole cells such as *Saccharomyces cerevisiae*, baker's yeast, as a broad specificity sensor. To take full advantage of the generality of such a microbial sensor, a thermistor was chosen as the most suitable transducer, since practically all reactions catalysed by the enzymes of a micro-organism are, in the right conditions, accompanied by tempera-

ture changes which can be registered by a thermistor. This is in marked contrast to the situation with bioelectrodes, where the electrode entity can only partly match the 'output' from the sensor because of its specificity for only one or a few different molecular species.

The yeast cells were immobilised in 15% polyacrylamide⁷ as follows: Washed baker's yeast cells (5 g wet weight) were suspended in 5 ml of ice-cold water, and mixed with 10 ml ice-cold freshly prepared monomer solution in 0.2 M potassium phosphate buffer, pH 7.0, containing 2.85 g acrylamide, 0.15 g *N,N'*-methylene-bis-acrylamide, 20 µl tetramethylethylenediamine and 10 mg ammonium persulphate. The mixture was immediately poured on to a glass plate provided with stainless steel spacers (0.7 mm), and another glass plate was placed on top of the liquid pool, thus forcing the suspension to form a layer 0.7 mm thick. Polymerisation started within 1 min and was considered complete after 1 h. The gel sheet obtained was passed through a stainless steel mesh giving gel grains of cubic shape, approximately 0.7 × 0.7 × 0.7 mm. Fines were decanted and the yeast-containing grains were finally washed in phosphate buffer for at least 5 h.

To test whether the yeast cells were viable after entrapment in polyacrylamide, the gel grains were disrupted mechanically using a spatula, and the released cells suspended in sterile water, counted under the microscope and spread on malt extract agar plates. Colony counting after incubation for 24 and 72 h at 30 °C revealed that approximately 6% and 8%, respectively, of the released cells had formed colonies. We conclude, therefore, that the entrapment leaves viable cells. It should also be realised, however, that non-viable cells contain several active enzymes⁸ and will therefore contribute to the heat formed in an operating microbe thermistor. The reduced viability of the entrapped cells was shown to be due to the toxicity of the monomers (15 min exposure of yeast cells to 15% monomer solution reduced the viability to 3%).

The microbe thermistor was identical to the enzyme thermistor unit described earlier⁴. Approximately 1 g of yeast gel grains were packed in an insulated glass column (0.6 × 4 cm) provided with a thermistor at its outlet. The solution to be analysed was pumped (0.7 ml min⁻¹) through a heat exchanger in an ultra-stable thermostatically controlled water-bath (27 °C) and then through the column. Any heat change occurring in the gel bed alters the resistance of the thermistor and thus gives a signal ultimately registered on a chart recorder (a change in the thermistor temperature of 0.02 °C gave a 100-mV change in the output signal at the highest amplification).

Some basic properties of the enzyme thermistor were deter-

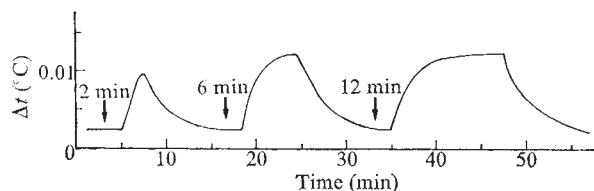
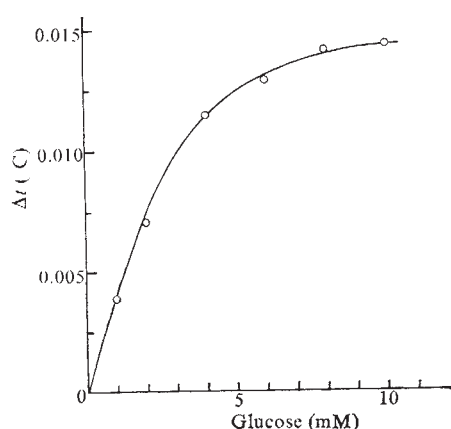


Fig. 1 Experimental curves obtained after injection of 1 mM glucose pulses (in 0.1 M potassium phosphate pH 7.0) of varying durations. Arrows indicate substrate introduction.

mined with glucose as substrate. Typical response curves shown in Fig. 1 are qualitatively quite similar to those observed with enzyme thermistors. On increasing the pulse length, an increased heat response was obtained until a constant level of heat production was reached. The correlation between heat production and glucose concentration (Fig. 2) is almost linear at low glucose concentrations, but at 10 mM and higher substrate concentrations a typical saturation behaviour is seen.

The microbe thermistor is thus of value for continuous analysis, but in its present state of development it cannot compete with enzyme electrodes, for example, with respect to speed of analysis. A faster response could probably be achieved by lowering the substrate/product diffusion time through the gel using surface-bound microbes and by increasing the substrate turnover choosing other microorganisms with higher metabolic activity. As expected, the microbe thermistor also proved to be sensitive to other compounds, although the response varied with the compound under study. Fructose (in 0.1 M potassium phosphate, pH 7.0) gave a heat signal approximately 60% of that found with an equal concentration of glucose, as well as a slower response and a faster decline back to the baseline. The high molecular weight substrate casein (1% in 0.1 M potassium phosphate buffer, pH 7.0) also gave a good response ($\Delta T = 0.014^\circ\text{C}$) but with a pronounced lag period before a steady-state level was reached. If, however, the casein was pre-degraded with Pronase, then a faster response was obtained, but with an unaltered final amplitude (measured after 30 min). The rate in the initial period was about twice as high in this case. Thus the entrapped cells can only degrade high molecular weight compounds with difficulty. The same phenomenon, but even more pronounced, was observed using 1% amylose, which gave no heat signal unless pretreated with amylase (batch- or in line enzyme column-wise). After hydrolysis the response was as fast as shown in Fig. 1. The model system studied here thus allows a differentiation between low and high molecular weight sugars. The preparations of entrapped yeast cells were stable for at least one month at 4°C and for about 7 d during continuous operating conditions, after which time clogging occurred.

Fig. 2 Measured peak height (ΔT in $^\circ\text{C}$) as a function of glucose concentration. The glucose substrate was dissolved in 0.1 M potassium phosphate and injected as 1-min pulses.



In another set of experiments, the sensitivity to various inhibitors was analysed (Fig. 3). Thus, on adding 2,4-dinitrophenol (1 mM) to the 1 mM glucose solution, an increased heat response was observed ($+20\%$). This may be interpreted as a result of the uncoupling of the oxidative phosphorylation, preventing conservation of metabolic energy (ATP) which in turn leads to an increased heat production. These results are analogous to the findings reported when dinitrophenol was added to a culture of the microalga *Scenedesmus*, being pumped continuously through a conventional flow microcalorimeter^{9,10}. On the other hand, 2 mM arsenate (which is known to influence the glyceraldehyde-3-phosphate dehydrogenase catalysed step) decreased the observed heat production from 1 mM glucose, as seen in Fig. 3.

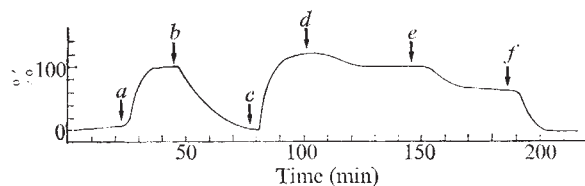


Fig. 3 Thermogram obtained with the microbe thermistor following introduction of various metabolites and inhibitors dissolved in 0.1 M potassium phosphate buffer pH 7.0 into the flow. The arrows indicate addition of: a, 1 mM glucose; b, buffer; c, 1 mM glucose + 1 mM 2,4-dinitrophenol; d, 1 mM glucose; e, 1 mM glucose + 2 mM arsenate; f, buffer. The steady-state response to 1 mM glucose is set as 100%.

The use of immobilised microorganisms in combination with suitable transducers offers a flexible alternative of great potential for the analysis of particularly complex solutions. Other organisms or alternative immobilisation techniques could be used in the microbe thermistor. In conclusion, the studies discussed here with the simple microbe thermistor device suggest the following potential areas for its application: (1) its use in analytical determinations of various single components (glucose, fructose) or of more complex solutions (casein); (2) its use in biochemical and microbiological studies, since it conveniently allows successive additions of, for instance, metabolic inhibitors (DNP) or media to the immobilised microbial preparation present in the flow system; and (3) it should also be useful in environmental and process control, as a general indicator for any compound that may affect the metabolism of the microorganism.

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Interspecific competition between great and blue tit

ALTHOUGH competition theory predicts that two species can coexist if intraspecific competition, between members of each species, is more important than interspecific competition, between members of different species, or if $\alpha < K_1/K_2$ and $\beta < K_2/K_1$ (with α and β competition coefficients and K_1 and K_2 carrying capacities for species 1 and 2), there were no animal field studies where these predictions are borne out. This letter, however, explains how two closely related titmice, although competing for food during the breeding season, coexist in broad leaved woods.

The great tit *Parus major* and the blue tit *Parus caeruleus* are two sympatric hole nesting passerines with deciduous forest as their optimal habitat. In winter, they consume different foods but during the breeding season they feed to a large extent on the same¹⁻⁴. Kluyver⁵ has, therefore, suggested twice that interspecific competition between the two tits might occur, but he provided no evidence for this. British authors^{2-4,6} on the other hand, have explained that this food niche overlap is possible because food would be superabundant during the breeding season and therefore competition would not occur. The data from the Ghent study (Table 1) show that great tit reproduction is significantly reduced both by higher great tit and by higher blue tit numbers. This is the result of a reduction of first brood

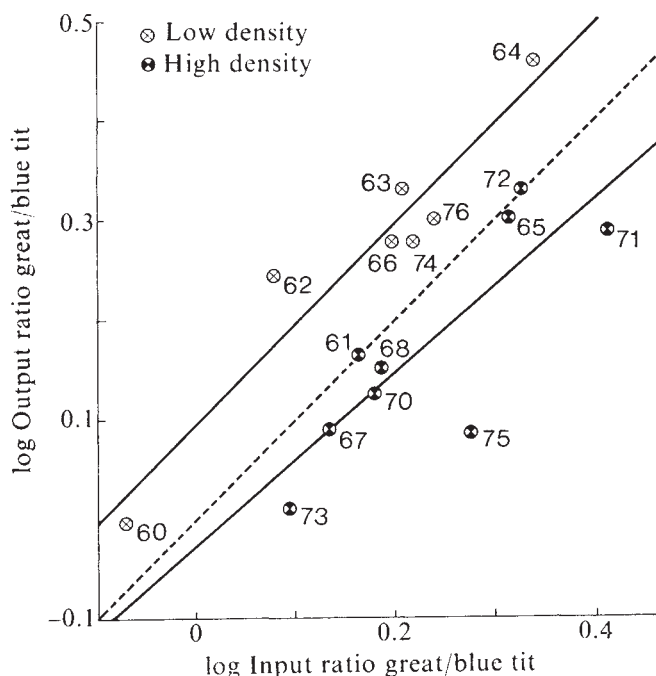
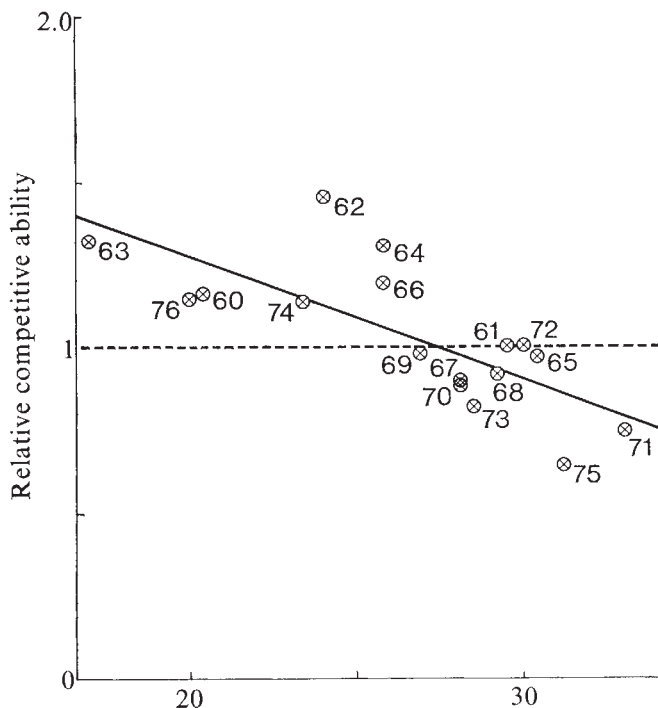


Fig. 1 In plant ecology, Dewit's experimental set up is used to compare the relative competitive ability of two species in different circumstances and at varying relative frequencies, but at the same combined density⁷. If the regression of log input ratio (here log density great tit/density blue tit) against log output ratio (here log number of young fledged great tit/blue tit) lies above the line with slope one through the origin, the species in the numerator does relatively better than the species in the denominator. If the regression line lies below this reference line the reverse is true. As this technique relies on experiments with the same combined density, I selected 7 yr with low combined densities (17–25.8 pairs per 10 hectares, open symbols), and 9 yr with high combined densities (28.1–33 pairs per 10 hectares, partly filled symbols). The low density line lies above, the high density line lies below the line with slope 1 through the origin (stippled), and their slopes are not significantly different from 1 (low density line: slope 1.01, $s_b = 0.14$; high density line slope 0.87, $s_b = 0.22$). This means that the relative frequency does not, but the combined density does affect the relative competitive ability of the tits.



Combined density great and blue tit per 10 hectare

Fig. 2 In Fig. 1, the years were grouped according to low or high combined density but since in this field study densities were not controlled this approach might be criticised. Because combined density seemed to be the factor determining which species will produce relatively more young, the relative competitive ability (ratio of great tit over blue tit density divided by ratio of great tit over blue tit total number of young fledged) is plotted against combined density. The correlation coefficient is highly significant ($r = -0.75$, $F_{1,15} = 19.18$, $P < 0.001$), confirming that the relative competitive ability is dependent on combined density.

clutches (strong intra-, weak interspecific effect), of an increase in nest mortality (strong intra-, strong interspecific effect), and of a reduction in the proportion of birds undertaking a second brood (strong intra-, and strong interspecific effect). Thus fewer young are produced per pair and per season (very strong intra- and very strong interspecific effect). Blue tit reproduction is affected in a similar fashion but the effect of the great tit is much less pronounced and is only statistically significant on nest mortality.

All these effects can be related to food. When less food is available per bird, because more birds of either species are present, clutches will be smaller, nest mortality will be higher and it will be more difficult for the females to make up for the weight losses incurred while raising their first brood and thus harder to start a second clutch. Although it could be argued that interference between the birds would have the same result this seems unlikely since (1) there are no observations to support that intra- or interspecific interactions are numerous during the nesting period and especially not when young are being raised (tits being highly territorial species), and (2) the effect of the larger great tit on the smaller blue tit should be more pronounced than vice versa, while in fact the opposite is the case.

Therefore, these negative effects of numbers on reproductive parameters are the result of intra- and interspecific competition for food of the scramble or exploitation type, rather than a possible result of interference competition. This leads to the conclusion that food cannot be superabundantly available during the breeding season.

Why then does the smaller and less numerous blue tit have a stronger effect on the larger and more numerous great tit, than the other way round? To explain this, I hypothesise that the niche of the great tit during the breeding season must be narrower and be contained to a large extent in the broader

Table 1 Intra- and interspecific effect on several reproductive parameters of great and blue tit

Parameter	Density great tit	Density blue tit	Multiple correlated coefficient	Partial correlated coefficient Blue tit constant	Partial correlated coefficient Great tit constant
Great tit					
Clutch size (1st brood)	-0.54*	-0.38	0.61*	-0.51*	-0.33
% second broods	-0.68**	-0.54*	0.80***	-0.70**	-0.57**
Fertility	-0.55*	-0.48°	0.67*°	-0.53*	-0.45°
Nest success	-0.55*	-0.56*	0.71**	-0.54*	-0.55*
Productivity	-0.69**	-0.64**	0.86**	-0.76***	-0.72***
Blue tit					
Clutch size (1st brood)	-0.42°	-0.54*	0.62*	-0.38	-0.51*
Fertility	-0.27	-0.32	0.38	-0.22	-0.28
Nest success	-0.45°	-0.65**	0.73**	-0.43°	-0.64**
Productivity	-0.39	-0.46°	0.55°	-0.34	-0.43°

The Ghent study was started by J. Hublé in 1959 and is at present supervised by R. Eyckerman. The data used are the weighted averages over the period 1960-1976, from the two best study areas in use since the beginning of the project—Zevergem, an oak wood of 16 hectares (13 hectares until 1963) with 65 nestboxes per 10 hectares, and Maaltepark, a mixed deciduous park of 10 hectares with 78 nestboxes (more details on the study areas in ref. 11). Thus nestboxes were always well in excess. Breeding parameters were determined from weekly controls of all nestboxes throughout the breeding season. Density is the number of breeding pairs per 10 hectares; mean clutch size is calculated from all complete clutches; fertility is the mean number of eggs laid per pair per season, including incomplete, repeat and second clutches; nest success is the proportion of eggs giving rise to fledged young; productivity is the mean number of young fledged from all nests per pair and per season. In our study areas nearly all great and blue tit pairs use nestboxes for breeding (personal observation, personal communication R. Eyckerman)¹².

° $P < 0.05$ * $P < 0.025$ ** $P < 0.01$ *** $P < 0.005$ **** $P < 0.001$ (one-tailed test).

feeding niche of the blue tit. The detailed observations in English deciduous forests seem to confirm this hypothesis: Gibb³ noted that during the breeding season blue tits feed to a large extent on twigs and buds, whereas the heavier great tit uses stouter parts of the tree. These are also used by the blue tit. Betts⁴ showed that Lepidoptera larvae, in May, make up the

bulk of the food for both species: 84% for the great tit and 56% for the blue tit. 41% of blue tit food (Lepidoptera pupae and Coccidae) is not taken by the great tit (and inaccessible?) whereas only 7% of the great tit diet (adult Coleoptera) is not taken by the blue tit.

This way, when it becomes scarce food inaccessible to great tits is still available for blue tits. It is thus shared with fewer competitors and will last longer.

The question that remains is how these two competing titmice coexist in the same preferred habitat. Dewit has shown that two plant species can coexist if the rarer species always has the competitive advantage⁷. Figure 1 shows, using an adapted version of Dewit's method, that the ratio of great versus blue tit numbers does not alter their competitive ability. However, at low combined densities the great tit has the competitive advantage, whereas at high combined densities the blue tit does better, and produces relatively more young. This suggests that the main factor influencing their relative competitive ability would be combined density. This is confirmed in Fig. 2. At a combined density of 27.5 pairs per 10 hectares the relative competitive ability is one and neither species has the advantage. At lower combined densities the great tit produces proportionally more young than the blue tit and at higher combined densities the reverse is true. As long as combined density fluctuates sufficiently both species will coexist. Since great tit numbers vary more than blue tit numbers this will probably result in a stable equilibrium: at low combined densities, great tit numbers will be small and they will increase more than blue tit numbers; this will result in high combined densities and great tits will decrease more than blue tits.

However, it is better to try and estimate competition coefficients and K -values. This is done in Fig. 3 and yields: $\alpha = 1.2$, $K_1 = 30$; $\beta = 0.13$, $K_2 = 13$ (1 = great tit, 2 = blue tit). Thus the effect of the blue tit on the great tit is much larger than vice versa confirming the result obtained in Table 1. The necessary conditions for a stable equilibrium are met since $1.2 < 30/13 = 2.3$, and $0.13 < 13/30 = 0.43$. This means that for both species intraspecific competition is more important than interspecific competition and that, therefore, the great and blue tit can coexist. The stable equilibrium point is at 17 pairs of great tit and 10.5 pairs of blue tit per 10 hectares. Remarkably, this gives a combined density of 27.5 pairs per 10 hectares, which was the stable equilibrium point found in Fig. 2. I suggest that intraspecific competition is mainly for space, as both species are known to be highly territorial and it has been shown that breeding numbers in optimal habitats, for the great tit at least, are limited through territorial behaviour

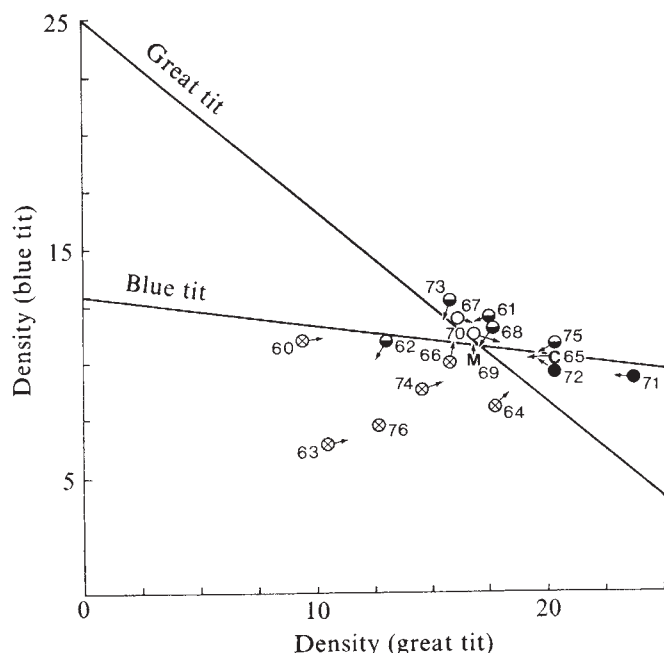


Fig. 3 Equilibrium lines for each species were estimated by requesting eight colleagues to fit the best line for each species so that the points showing an increase until the next year would be below it, the points showing no change on it, and the other points above it. The lines drawn are the averages of these estimates. Although the equilibrium lines varied, in all cases they intersected in such a way that a stable equilibrium would be obtained. The point for 1962 was disregarded because the winter 1962/63 was extremely cold and mortality, especially of the juveniles, was unusually high. The intersections of the equilibrium lines with the axes give: for the great tit $K_1 = 30$, $K_1/\alpha = 25$, and $\alpha = 1.2$; for the blue tit $K_2 = 13$, $K_2/\beta = 100$, and $\beta = 0.13$. ⊗, both species increase; ⊙, both species decrease; ●, blue tit increases, great tit decreases; ⊖, great tit increases, blue tit decreases; M: great tit unchanged, blue tit increases; C: blue tit unchanged, great tit decreases.

(in spring and in autumn)⁸⁻¹⁰, whereas interspecific competition is mainly for food during the breeding season.

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Cercal ablation modifies tethered flight behaviour of cockroach

ANALYSES of insect flight demonstrate a central rhythmic programme modified by means of cephalic and thoracic receptors to allow steering and power output adjustments¹⁻³. In orthopteran insects, cercal receptors have never been implicated in flight control, although consideration of their function in cockroaches as directional air current detectors⁴ makes this rather surprising. I report here that removal or inactivation of one cercus in a tethered flying cockroach leads to asymmetrical wing movements during flight. Removal of both cerci causes inactivation of forewings and symmetrical beating of both hind wings at a reduced angle to the abdomen. These results suggest cockroach cerci function as specialised equilibrium organs and may relate to the function of cercal interneurons perhaps including the well studied giant fibres (for which there is now no suggested function⁵).

The proterga of unanaesthetised cockroaches (*Periplaneta americana*) were attached by sealing wax to an orthogonal system of three threads which were each further attached to the metal spring of a resistive strain gauge transducer. This allowed limited translation and rotation of the cockroach in three dimensions (Fig. 1). Flight is a complicated behaviour pattern, but for the purposes of this report it is sufficient to describe the approximate angles of the wings with respect to the body and the occurrence or non-occurrence of rhythmic beating. It is unnecessary to present detailed strain gauge measurements. All angles are measured clockwise relative to the median sagittal plane of the suspended cockroach seen in frontal view.

Suspended cockroaches would fly spontaneously, or could be made to fly by a short puff of air to the head; by making and then breaking tarsal contact with, for example, a finger, or by forced oscillation of the supporting threads. They would often fly for 2 or 3 min. Only one out of 13 cockroaches used did not fly well. After ether anaesthesia in early experiments, cockroaches required 3 d for recovery before they flew well. In normal symmetrical flight (Fig. 2a), forewings are held at approximate angles of 90° and 270° for right and left wings respectively and oscillated rhythmically. Hind wings are held at angles of about 120° or 240° and also oscillated. If the threads are asymmetrically fixed to the protergum, so that the cockroach is suspended asymmetrically then the wings on the down-tilted side are held out and oscillated for a longer time than the wings on the other side, and the down-tilted side

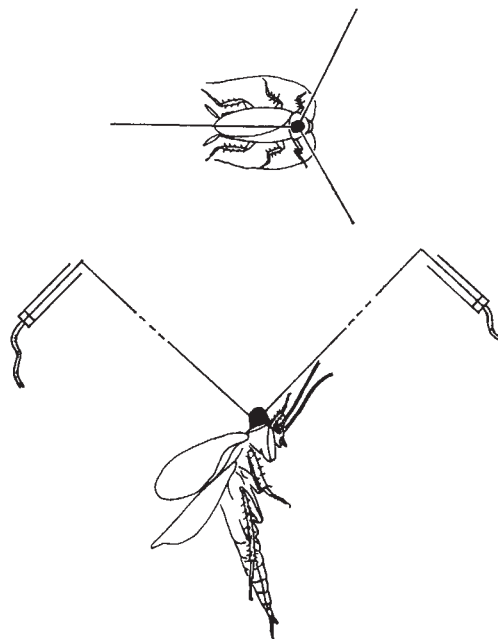


Fig. 1 Dorsal and lateral diagrammatic views of cockroaches suspended by threads from the springs of strain gauge transducers arranged orthogonally. Not to scale.

forewing tends to remain out after all wing oscillation has stopped. If weak flight is initiated, the wings on the uptilted side are not used. If stronger flight is initiated, all four wings are extended and oscillated, but the hind wings on the uptilted side are held closer to the abdomen at angles of 160° or 200°, very near their closed position. As flight continues, the up-tilted side wings tend to be folded away while the down-tilted side wings continue to beat. Compensatory displacements of leg and abdomen are also produced. The effects of tilting are similar to those produced by single cercus ablation (Fig. 2b, c and d).

Removal of one cercus causes similar asymmetrical flight in a previously symmetrical flier. Thus when the right cercus is removed (Fig. 2b, c and d) the wings on the right side continue to be held and oscillated during flight as for normal symmetrical flight in the intact animal. The hind wing on the left side, however, is held and oscillated near its closed position at an angle of 200°, and in time, ceases to be used. The forewing, may be held extended and oscillated at first, but it is soon withdrawn, leaving the hind wing oscillating. The main torque is exerted by the right wings and will tend to turn the cockroach clockwise (seen from the front). Compensatory movements of abdomen and legs consisting of a tilting of the abdomen to the left and extension of left legs and flexion of right legs are usually apparent. These compensatory movements will tend to turn the animal in the same direction as torque from the wings.

Removal of both cerci abolishes normal tethered flight (Fig. 2e). Forewings remain folded, but hind wings are held at angles of 160° and 200° and oscillated.

These reflexes were highly stable and easily evoked. When a cockroach which was asymmetrically suspended so that it tended to beat its left wings only, had its right cercus removed, it then beat its right wings in the normal flight manner and ceased to use its wings on the left. It was not necessary to remove the cerci to evoke these reflexes. Interference with the sensory hairs by smearing with mineral jelly caused asymmetrical flight as if the inactivated cercus had been removed. After removal of the jelly, flight was symmetrical as before.

It seems clear that the cerci should be regarded as equilibrium organs which control equilibrium and compensatory reflexes important in flight. This suggested equilibrium function is not incompatible with existing suggested functions for cerci as auditory organs, vibration receptors or air movement detectors⁴. Bischof on electrophysiological evidence has assigned a gravity

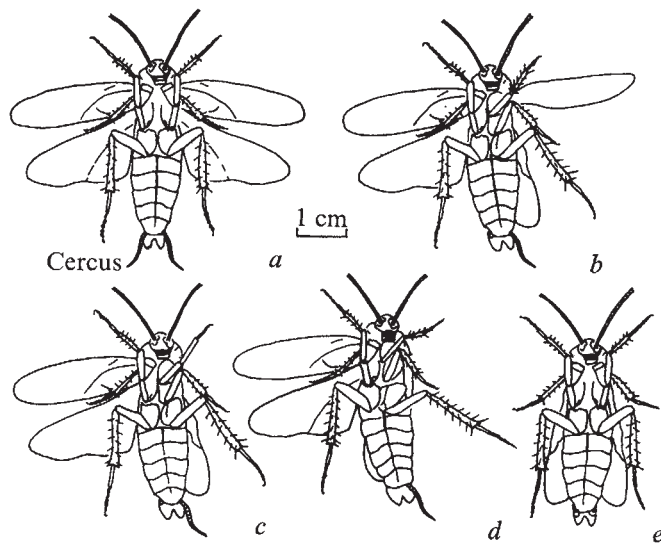


Fig. 2 Frontal views of typical wing and leg positions during tethered flight. *a*, Intact, symmetrically suspended cockroach; *b-d*, right cercus removed showing successive lack of use of left wings; *e*, both cerci removed. Hind wings only are oscillated and are not fully extended.

reception function to club-shaped hairs on the cerci of a cricket⁶. The simplest explanation for the effects reported here is the gravity receptors on cockroach cerci control equilibrium reflexes involving wings and legs in an analogous way to the control of eyestalk and appendage movements by the crustacean statolith⁷. But during inherently unstable locomotion such as flying, an angular movement detector might be expected, analogous to the semicircular canal system of crabs^{7,8}, facilitating rapid activation of compensatory reflexes. The directional wind sensitive hairs of the cerci could function in this way to detect changes in the velocity of air relative to the cerci which would be caused by angular displacement of the cockroach during forward flight (although many other widely distributed mechanoreceptors, could provide such information⁹).

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Transfer of mitochondria by protoplast fusion in *Saccharomyces cerevisiae*

INTRASPECIFIC and interspecific transfer of nuclear genes of fungi has recently been achieved by fusion of protoplasts¹⁻⁵. We describe here the transfer of mitochondrial genome using the protoplast fusion technique. The experiments were carried out with strains of identical mating-type, so we also report successful complementation of auxotrophic yeast cells of the same mating-type after protoplast fusion.

The main steps of the experiments were as follows: (1) selection of a mitochondrial donor with stable nuclear and mitochondrial markers, and of a recipient with a nuclear colour marker and p^0 petite mitochondrial character. In both cases it

was an additional requirement to have strains of identical mating-type to exclude the possibility of mitochondrial transfer by zygote formation; further, rapid and complete protoplast formation, followed by high-frequency reversion to intact cells, was also a basic necessity. (2) Protoplast formation. (3) Induction of fusion of donor and recipient protoplasts and selective regeneration of those undergoing complementation. (4) Characterisation of the fusion products. And (5), recovery of the recipient containing the marked mitochondria of the donor.

The uracil and tryptophan-requiring strain IL 216-1B (nuclear genotype: α *ural*, *trp1*; mitochondrial genotype: ω^+ E_{291}^R) was selected as mitochondrial donor. The oxygen-consumption of log-phase cells in yeast extract-glucose medium was 21.8 μ l O_2 per mg wet weight per h. This strain can grow on non-fermentable carbon sources in the presence of a high concentration (5 mg ml^{-1}) of erythromycin. A p^0 petite mutant of the erythromycin-sensitive (0.2 mg ml^{-1}) adenine-requiring grande strain DV 147 (nuclear genotype: α , *ade2*) was selected as mitochondrial recipient. In the petite mutant no mitochondrial DNA was detected by sensitive fluorescent staining techniques using 4',6-diamidino-2-phenylindole (DAPI) (refs 6, 7) or 2-[2-(4-hydroxyphenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl) benzimidazole.3HCl (33258 Hoechst)⁷ in CsCl gradients. The recipient showed no respiration and was unable to grow on non-fermentable carbon sources or galactose. On minimal medium supplemented with adenine (optimum 5 μ g ml^{-1}), the grande DV 147 strain formed fast-growing red colonies, while the colonies of the petite mutant were slow-growing and white.

For protoplast formation the cells were cultivated in yeast extract-glucose medium, collected in the early log-phase, treated with 1% 2-mercaptoethanol⁸ for 10 min, collected by centrifugation, resuspended in 0.7 M KCl containing 0.1% 2-mercaptoethanol and 1% snail enzyme to give a final cell concentration of 10^8 ml^{-1} , and gently shaken at 30 °C. All the cells were converted into protoplasts in 1 h; these were collected and washed twice with 0.4 M $CaCl_2$ by centrifugation, and the two strains were then mixed in a 1:1 ratio (5×10^7 protoplasts each). The fusion process was carried out with 35% polyethylene glycol (PEG, molecular weight 4,000) as described previously^{2,9}. The suspension of aggregated protoplasts was diluted with 35% PEG, mixed at 43 °C with vitamin-containing¹⁰ minimal medium supplemented with 0.4 M $CaCl_2$ and 2% agar, and poured as a thin top layer on to a similar medium. In this medium, only fusion products complemented nutritionally by heterokaryon or diploid (polyploid) formation between the two partners taking part in the fusion process can regenerate and grow into colonies of wild phenotype.

No colonies appeared if intact cells or protoplasts of only one of the partners were applied; if intact cells of the two partners were cultivated together for mating; or when no PEG treatment was used for aggregation of protoplasts. This means that no back-mutation was observed; no complementation occurred with intact cells of identical mating type; and PEG treatment is essential to induce the fusion process of yeast protoplasts. On the other hand, good regeneration and colony growth was observed in all these cases in nutritionally-sufficient agar media.

The complementation frequency proved to be about 0.04% if all protoplasts taking part in the fusion process were considered, and about 0.6% if the number of colonies growing in minimal medium was compared with the number developing in nutritionally-rich medium. In the present fusion system, the frequencies obtained were similar to those not only for other $\alpha + \alpha$ or $\alpha + a$, but also for $a + a$ pairs of protoplasts (unpublished), indicating that the protoplast fusion is not affected by mating factors.

The main characteristics of the nutritionally-complemented and regenerated fusion products were as follows. On or in minimal and rich media their cultures, always white, grow more intensively than the fusion partners in optimum conditions, with increased formazan production if tetrazolium violet (20 μ g ml^{-1}) was applied in the media. These properties are indications

of enhanced metabolic processes. In the fusion products only one nucleus per cell was demonstrated by staining with acridine orange¹¹. The average cell size of the fusion products was significantly larger than that of either of the partners. The sizes and DNA determination data are given in Table 1. DNA was extracted by the Bostock procedure¹² and estimated with diphenylamine^{13,14} using chicken erythrocyte DNA as standard. A bracketed symbol [x + y] is proposed for the fusion products, at least until they have been characterised thoroughly.

Table 1 Cell sizes and DNA contents of the fusion partners, fusion products, and erythromycin-resistant haploid and aneuploid segregants.

Strains	Length (µm)	Width (µm)	DNA content (fg DNA per cell)
IL 216-1B p ⁺ E ^R	5.37±0.14	4.82±0.12	24.4±1.8
DV 147 p ⁰	4.73±0.16	4.43±0.13	22.5±2.3
[IL 216-1B p ⁺ E ^R + DV 147 p ⁰]	7.57±0.15	5.42±0.15	48.0±4.1
DV 147 p ⁺ E ^R (haploid)	5.62±0.17	5.30±0.16	22.5±0.3
DV 147 p ⁺ E ^R (aneuploid)	5.55±0.14	5.04±0.13	30.7±0.6

Values are ± s.e.m. Standard errors are based on 50 measurements and at least three DNA determinations.

These data, together with the observation that the fusion products can be induced to undergo haploidisation, suggest that the fusion products were diploids. No spore formation and only rare spontaneous haploidisation, with a frequency of less than 10⁻⁴, has yet been demonstrated. All fusion isolates were resistant to erythromycin at the same level as the donor strain.

By induced haploidisation with *p*-fluorophenylalanine¹⁵⁻¹⁶ (400 to 600 µg ml⁻¹) or with benomyl¹⁷⁻¹⁸ (6–15 µg ml⁻¹) in yeast extract-glucose medium, up to 40% adenine-requiring haploid and aneuploid red colonies with α genome could be isolated. These colonies consisted of respiration-sufficient cells harbouring mitochondrial resistance to erythromycin, indicating successful mitochondrial transfer. Among the resulting adenine-requiring E^R isolates, several proved different from the original DV 147 strain in certain properties. One was the generation of a much lower amount (< 2%) of spontaneous petites, in contrast to the case with the original strain (up to 60%). These properties are under investigation.

Mitochondrial transfer by protoplast fusion—'transfusion' of mitochondria—may contribute considerably to mitochondrial biochemistry and genetics. Not only intraspecific, but also interspecific mitochondrial transfer and recombination of mitochondrial DNA of distant species seems feasible and this possibility is currently under study. It additionally seems of interest to exploit the possibility of fusion of protoplasts of identical mating-type and in this way to build up various α, α or specially mixed nuclear genomes for basic research and practical applications, by overcoming the mating-type barrier.

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Circular linkage map of *Rhizobium meliloti* chromosome

GENETIC studies on symbiotic nitrogen-fixing rhizobia are greatly hampered by the lack of an efficient system for genetic mapping in rhizobia. Although there have been reports on transformation¹⁻³ and transduction⁴ in nodulating rhizobia as well as conjugation⁵ in a non-nodulating strain of *Rhizobium lupini*, the genetic map of a nodulating and nitrogen-fixing *Rhizobium* strain has not been established yet. Plasmids of the P-1 incompatibility group have a remarkably wide host range and can be transferred to many *Rhizobium* species^{6,7}. Some of these plasmids can promote chromosome transfer in *Pseudomonas aeruginosa* strain PAT but show little chromosome donor ability (Cda) in strain PAO^{8,9}. Recently a variant (R68.45) of the P group plasmid R68, with high Cda property in strain PAO, was isolated by Haas and Holloway¹⁰. Plasmid R68.45, unlike most of the sex factors, promotes chromosome transfer for a range of chromosomal genes in *Rhodospseudomonas*, *P. putida*, *Escherichia coli* (B. W. Holloway, personal communication) and in *Rhizobium leguminosarum*¹¹. We have found that this plasmid can promote chromosome transfer also in *Rhizobium meliloti* and can be used for mapping of the chromosome. We present here a circular genetic map of the *R. meliloti* 41 chromosome and describe a new approach in the evaluation of conjugational mapping data which may be applicable to other Gram-negative bacteria.

Plasmid R68.45 was introduced into *R. meliloti* 41 strains AK208 and AK209, and the chromosome donor ability (Cda) of the product was investigated. Various auxotrophic and antibiotic resistance markers were used as chromosomal markers in plate matings on nutrient agar¹². As seen in Table 1, R68.45 could be transferred between *R. meliloti* strains at frequencies of (3–7) × 10⁻¹ per donor cell and recombinants for various markers were produced at frequencies of 10⁻³ to 10⁻⁵ per donor cell. This frequency of recombination is about the same as that found in *P. aeruginosa* PAO¹⁰ and about 2–3 orders of magnitude higher than in *R. leguminosarum*¹¹. Recombination

Table 1 Plasmid transfer and chromosome mobilisation by R68.45 in *R. meliloti*

Donor	Recipient	Frequency of Tc ^R transconjugants	Selected chromosomal marker	Recombination frequency
AK208	AK80	6.3 × 10 ⁻¹	<i>cys46</i> ⁺	3.6 × 10 ⁻⁴
			<i>str1</i>	1.9 × 10 ⁻⁴
AK208	AK193	5.2 × 10 ⁻¹	<i>gly1</i> ⁺	2.8 × 10 ⁻⁴
			<i>met2</i> ⁺	2.6 × 10 ⁻⁴
AK209	AK80	3.2 × 10 ⁻¹	<i>cys46</i> ⁺	1.2 × 10 ⁻⁴
			<i>str1</i>	8.0 × 10 ⁻⁵
AK209	AK193	4.4 × 10 ⁻¹	<i>gly1</i> ⁺	1.3 × 10 ⁻⁴
			<i>met2</i> ⁺	1.5 × 10 ⁻⁴

Mating conditions in all experiments were as described before¹². Selective medium: minimal medium (GTPS¹³) was supplemented with amino acids, 50 µg ml⁻¹; bases, 25 µg ml⁻¹; streptomycin, 100 µg ml⁻¹; rifampicin, 100 µg ml⁻¹; tetracycline-HCl, 15 µg ml⁻¹, as required. Agar surface matings were carried out for 16 h at 37 °C. Bacteria were scraped off the surface of the plates, resuspended in saline and plated on selective media. Counterselection for AK208 and AK209 was by *his1* and *ura29*, respectively. Strains are described in Table 2. Transfer frequency: Tc^R transconjugants per donor bacterium. Recombinants on same basis.

Table 2 Linkage analysis of various marker pairs of *R. meliloti*

Donor	Recipient	Marker pairs	Linkage <i>c</i>	Distance <i>d</i>	Additive distance
AK213	AK80	<i>cys46</i> ⁺ <i>rif1</i>	0.10	0.54	0.64
		<i>str1</i> <i>rif1</i>	0.73	0.10	
AK213	AK193	<i>cys46</i> ⁺ <i>str1</i>	0.04	0.66	
		<i>cys46</i> ⁺ <i>rif1</i>	0.16	0.46	
		<i>met2</i> ⁺ <i>rif1</i>	0.05	0.63	0.40
AK208	AK193	<i>gly1</i> ⁺ <i>rif1</i>	<0.01	—	
		<i>cys46</i> ⁺ <i>met2</i> ⁺	<0.01	—	
		<i>gly1</i> ⁺ <i>ade4</i> ⁺	0.75	0.09	
		<i>ade4</i> ⁺ <i>met2</i> ⁺	0.33	0.31	0.16
AK208	AK194	<i>gly1</i> ⁺ <i>met2</i> ⁺	0.22	0.40	
		<i>tyr1</i> ⁺ <i>gly1</i> ⁺	0.79	0.08	
		<i>gly1</i> ⁺ <i>ade4</i> ⁺	0.77	0.08	0.16
AK208	AK184	<i>tyr1</i> ⁺ <i>ade4</i> ⁺	0.66	0.13	
		<i>gly1</i> ⁺ <i>leu4</i> ⁺	0.06	0.61	

Strains: AK80: *cys46* (F46 of ref. 14); by successive mutagenesis with nitrosoguanidine (NTG)—AK193: *cys46*, *gly1*, *ade4*, *met2*, *str1*; AK194: *cys46*, *gly1*, *tyr1*, *str1*; AK184: *cys46*, *gly1*, *leu4*, *str1*. AK213: *his1*, *str1*, *rif1* (R68.45) by transconjugation of a spontaneous *rif1* mutant of AK74, *his1*, *str1* (A1 of ref. 13)—AK208: *his1*, *str1* (R68.45) by transconjugation of AK74. Counterselection for donors by *his1*; selected for single markers, other marker by replica plating, averaged value for both directions. Strains used in other Tables: AK171 and AK173: *met33* and *ura29* mutants by NTG from ZS1, an *ade1*, *str1* mutant of Z. Sváb; AK209: *ade1*, *ura29*, *str1* (R68.45) by transconjugation of AK173; GY23: *cys46*, *gly1*, *leu4*, *str1* (R68.45) by transconjugation of AK184; AK189: *cys46*, *gly1*, *ade4*, *str1* (R68.45) by transconjugation of plasmid into a two-step NTG mutant of AK80.

frequencies for various markers were essentially the same when different donors were used, so this suggests that R68.45 promotes gene transfer from several chromosomal sites in *R. meliloti*.

We found that R68.45 can lose its Cda property during growth but other properties of the plasmid, such as resistance and transfer markers, are stably inherited. Therefore special care has to be taken to maintain effective donor cultures, as suggested by Haas and Holloway¹⁰. Recombinants selected for various chromosomal markers were stable but in many instances (10–80%) plasmid markers (TcR or Cda) were partially or completely lost in the recombinants, irrespective of the chromosomal marker selected. These observations also indicate that plasmid R68.45 behaves in *R. meliloti* 41 as it does in *P. aeruginosa* PAO. In recombinants selected for given chromosomal marker and possessing intact R68.45, R68.45 promoted the transfer of the given marker and of various other markers at the same frequency. This suggests that R68.45 is not integrated into the chromosome of the recombinants. Furthermore, using mutants of *E. coli* we demonstrated that the appearance of recombinants after chromosome mobilisation by R68.45 requires *recA* function, as was expected.

Double or multiply-marked derivatives of *R. meliloti* 41 were mated with derivatives of *R. meliloti* carrying R68.45. Recombinants for single markers were selected and coinheritance of unselected markers was determined by replica plating. The linkage data for certain marker pairs are shown in Tables 2 and 3. In repeated experiments the linkage frequency (number

of colonies with the phenotype of both selected and unselected markers per colony showing the phenotype of the selected marker) for a given marker pair varied by up to 10%.

Taking linkage (or cotransfer) frequency to be inversely related to the distance between genes, one usually obtains enough information to establish the marker order, but values of cotransfer frequencies are not additive. To establish a genetic map with additive map distances we attempted to transform our linkage data into additive map distances. We found that the map function derived by Wu¹⁵ for transduction was satisfactory for relating our linkage frequency values (*c*) to the map distance (*d*) (in arbitrary units) between two markers

$$c = (1-d)^3$$

We calculated and compared the *d* values and their additivity, for given marker pairs, obtained in separate experiments and in three-factor crosses. Examples for additive distance values, calculated in three-factor crosses, are shown in Tables 2 and 3. Experimentally determined *d* values are summarised in Fig. 1. Within the limits of our present experimental data, the correlation is fairly satisfactory for *c* values higher than 0.1.

There is not enough information about the mechanism of chromosome transfer by R68.45 to justify the use of Wu's equation, derived from the assumption that in transduction the donor fragments have nearly constant length and various regions of this fragment are incorporated into the chromosome of the recipient by pairs of crossover events. Nevertheless,

Table 3 Linkage analysis of the *cys46* region in *R. meliloti*

Donor	Recipient	Counterselected/ selected markers	Unselected classes of recombinants (%)*			Linkage frequency of pairs, <i>c</i>			Map distance <i>d</i>	Additive distance		
GY23	AK171	<i>gly1/ade1</i> ⁺	<i>cys46</i>	<i>met33</i>	51	<i>ade1</i> ⁺	<i>cys46</i>	0.62	0.15	0.59		
			<i>cys46</i> ⁺	<i>met33</i>	38	<i>cys46</i>	<i>met33</i> ⁺	0.18	0.44			
			<i>cys46</i>	<i>met33</i> ⁺	11	<i>ade1</i> ⁺	<i>met33</i> ⁺					
		<i>gly1/met33</i> ⁺	<i>cys46</i>	<i>ade1</i>	8	<i>met33</i> ⁺	<i>cys46</i>	0.14	0.48	0.13		
			<i>cys46</i>	<i>ade1</i> ⁺	6	<i>met33</i> ⁺	<i>ade1</i> ⁺	0.06	0.61			
			<i>cys46</i> ⁺	<i>ade1</i>	85	<i>cys46</i>	<i>ade1</i> ⁺					
		<i>cys46/met33</i> ⁺	<i>leu4</i>	<i>gly1</i> ⁺	28	<i>met33</i> ⁺	<i>leu4</i>	0.28	0.35	—		
			<i>leu4</i> ⁺	<i>gly1</i> ⁺	72	<i>met33</i> ⁺	<i>gly1</i>	<0.01				
			AK189	AK173	<i>gly1/ade1</i> ⁺	<i>cys46</i>	<i>ura29</i>	50	<i>ade1</i> ⁺		<i>cys46</i>	0.58
		<i>cys46</i>				<i>ura29</i> ⁺	8	<i>ade1</i> ⁺	<i>ura29</i> ⁺	0.08	0.57	
<i>cys46</i> ⁺	<i>ura29</i>	42				<i>cys46</i>	<i>ura29</i> ⁺					
<i>gly1/ura29</i> ⁺	<i>cys46</i>	<i>ade1</i>			11	<i>ura29</i> ⁺	<i>cys46</i>	0.20	0.41	0.14		
	<i>cys46</i>	<i>ade1</i> ⁺			9	<i>ura29</i> ⁺	<i>ade1</i>	0.09	0.55			
			<i>cys46</i> ⁺	<i>ade1</i>	80	<i>ade1</i> ⁺	<i>cys46</i>					

Strains and markers: see Table 2.

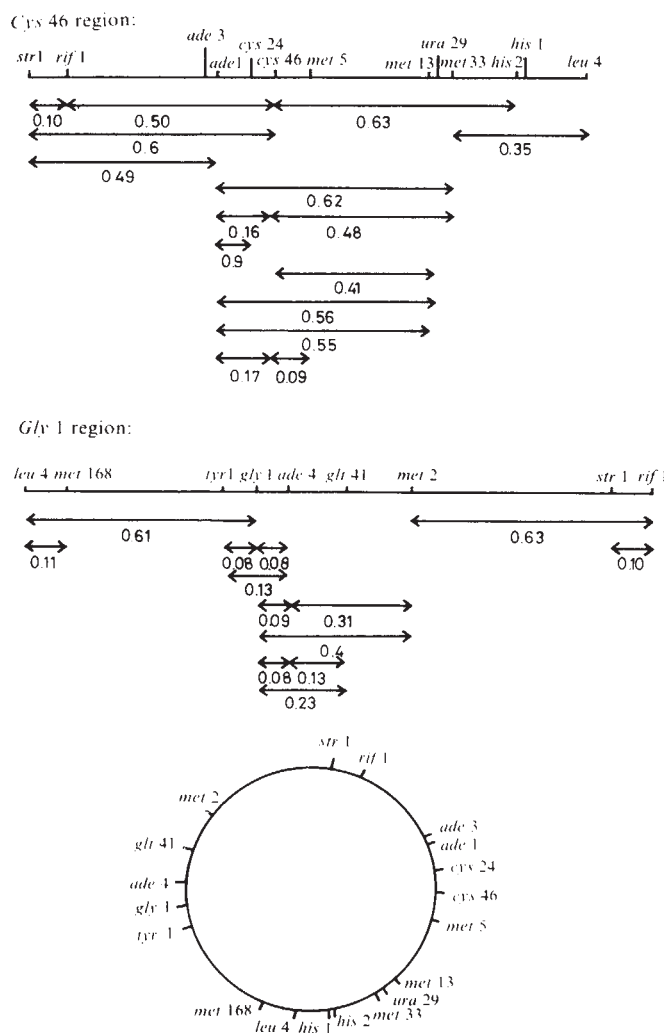
^{*}Unselected marker classes not given were less than 1%.

our data do indicate that the equation is applicable for converting linkage frequencies into approximatively additive map distance values.

The order of markers was determined by calculating map distances between closely linked markers or by three-factor crosses. Data in Table 2 indicate the order *tyr1-gly1-ade4-met2-str1-rif1-cys46*. Examining the linkage of unselected markers in three-factor crosses gave the linkage data shown in Table 3. From all of these results the order *str1-ade1-cys46-met33-ura29-leu4-gly1* could be deduced. The results also placed the additional markers *cys24*, *met5*, *met13*, *his1*, *his2*, *met168* and *glt41* in relation to the others. By confirming the linking of both ends of the above sequences, a circular map could be established (Fig. 1). The total length of the chromosome is about 3.1–3.2 arbitrary units (*d*). Mutant strains with single markers are being mapped in our laboratory. Using our multiply-marked mutants five matings are enough to locate any mutation on the linkage map.

In the original map function derived by Wu, $c = (1-d/l)^3$ the average size of the transducing particle (*l*) was known from independent measurements. Applying this equation for our system and using the information that the length of the chromosome is 3.2 *d* units, the average size of the donor fragment could be calculated. This is about 1/3 of the chromosome. On the other hand, however, in several cases we could demonstrate 0.05–1.0% co-inheritance of markers up to 1.5 *d* units apart (data not shown). This suggests that about 1% of the donor fragments are at least halves of the total chromosome in size.

Fig. 1 Linkage map of *R. meliloti* 41. Distances between markers are *d* values, obtained directly from independent crosses. Locations of *cys46*, *met5*, *met13*, *his1*, *his2*, *met168* and *glt41* were determined in three-factor crosses.



Therefore we could not calculate *d* values from linkage frequency values (*c*), lower than 0.03.

In conclusion, our data indicate that R68.45 is suitable for genetic mapping in *Rhizobium meliloti*. The mapping was based on linkage analysis of chromosomal markers and a circular map of the *R. meliloti* 41 chromosome was constructed. Recently Meade and Signer demonstrated linkage of chromosomal markers of *R. meliloti* 2011, using RP4 as a sex factor¹⁶. Using R68.45 we are directing our efforts to map those genes which are involved in symbiotic nitrogen fixation.

Although we do not have independent evidence to support our empirical use of the described map function relating linkage frequency to the map distance, we suggest that the map distances between markers are approximately proportional to the physical marker distances around the chromosome. Preliminary experiments in our laboratory indicate that this equation is applicable for linkage frequencies, obtained with R68.45, in *E. coli* as well. Furthermore, this plasmid would be useful for genetic mapping in many other Gram-negative bacteria, and our new approach for evaluating conjugational mapping data will certainly be useful for these future genetic systems.

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Note added in proof: Drs J. E. Beringer and A. W. Johnston have informed us that they have established a circular linkage map of *R. leguminosarum* chromosome¹⁷ and have demonstrated interspecific recombination mediated by R68.45 among various *Rhizobium* species¹⁸. A linkage map of *R. meliloti* has recently been reported by Meade and Signer¹⁹.

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Very close linkage between HLA-B and Bf inferred from allelic association

THE human leukocyte antigen loci (HLA, previously HL-A) are known to be closely linked to the locus defining factor B of the alternative complement pathway¹. The B-factor locus was previously termed GBG (glycine-rich β -glycoprotein) and is now known as Bf. If the consequences of recombination do not impair survival we may use data on allelic association (sometimes termed linkage disequilibrium) as a rough estimate of the upper limit of the recombination fraction, θ . This approach has advantages

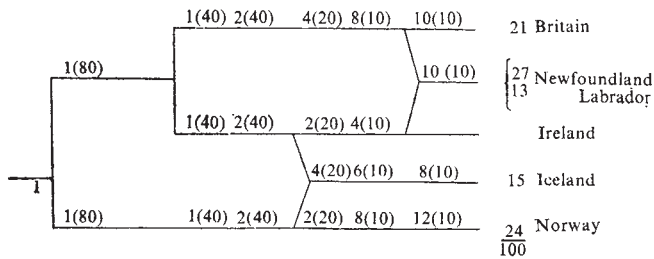


Fig. 1 A 'family tree' of the populations studied. Dimensions are time in generations (in brackets) and assumed numbers of ancestral B8 loci (minimum plausible estimates).

where linkage is very close and the frequency of laboratory and other errors in ascertaining recombinants is greater than the recombination frequency. By typing two or more members of families we have defined 100 apparently independent haplotypes with the HLA-B allele B8 and all had the Bf allele S. These observations were fairly evenly divided between Iceland, north-west Newfoundland and Labrador, Central England and Norway. Figure 1 shows a 'family tree' of these populations.

Iceland was settled in AD 874 by Norsemen with Irish slaves and had a population of over 70,000 by AD 1100; the Nordic and Celtic genetic contributions seem to have been comparable². Newfoundland was settled more slowly, but was substantially settled over 200 yr ago, mainly from England and Ireland. There are few data on the time of separation of the main Celtic, Saxon and Norse groups, or on the scale of the Saxon invasions.

The dimensions of the tree are time in generations (in brackets) and inferred minimal numbers of ancestral haplotypes. The number of individuals bearing the haplotype who were the ancestors of the tested population cannot be known, but can never have been greater than in the tested population. At some point in history all the tested populations had common ancestors and, in the remote past, there must have been a common ancestor of all the tested individuals. We feel that the dimensions of the tree are not unreasonable and are intentionally highly conservative, as is the assumption of a single ancestral haplotype at 3000 BC.

There are no apparent recombinants between the B and Bf loci in our sample. The chance of none of c haplotypes recombining in n generations is $(1-\theta)^{cn}$. The chance of an S allele being replaced by another is the relative proportion of these others, which is about a third in our population, so that the chance of no evident recombinant is $(1-\theta/3)^{cn}$. According to our assumptions, cn over all links is about 2,000. The table shows the probability of B8 alleles being exclusively associated with S on assumptions of various recombination fractions, assuming no selection against recombinants. While mild selection against recombinants will explain a substantial deficiency of B8-F haplotypes, a very strong selective disadvantage would have to be postulated to explain the complete absence of contemporary recombinants in such extensive data.

Table 1 Probabilities of no detectable recombinant in 1,500 and 3,000 meioses

θ	1,500	3,000
0.001	0.60	0.37
0.002	0.37	0.14
0.003	0.22	0.05
0.004	0.14	0.02
0.005	0.08	0.01
0.006	0.05	
0.008	0.02	
0.010	0.01	

We are aware of slight selective bias in selecting the B8 allele for special consideration; however, this allele is of particular interest for other reasons. One B8-F₁ haplotype has been reported by Hauptmann *et al.*³. This variant is usually associated with B18 in our experience. Albert *et al.*⁴ also found a complete and significant deficiency of B8-F (0/30) haplotypes in Germany, where Bender *et al.*⁵ found 4 B8-F haplotypes, 1 B8-S₁ and 64 B8-S, and also reported a B18-F₁ haplotype. B18 is also allelically associated with deficiency of the second component of complement (C2).

We consider our data to be evidence of very close linkage between the loci of B and Bf. If the determinants relating to graft survival are stronger at the B locus or are determined by loci which are closer to this locus than to the A locus, Bf typing might be of more practical value than HLA-A typing. It may also provide *a priori* evidence suggestive of a serologically definable split when one B allele is commonly associated with more than one Bf allele.

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Factors from 3T3 cells stimulate proliferation of cultured vascular endothelial cells

TUMOURS induce vascularisation into the neoplasm to provide needed cell nutrients and remove wastes¹. The formation of tumour blood vessels seems to depend, in part, on the proliferation and movement of vascular endothelial cells which are, in turn, controlled by the secretion of tumour angiogenesis factor (TAF) (refs 2-4). Extracts of tumours²⁻⁴ and of established cell lines⁵ have TAF activity *in vivo*. But little is known about the molecular nature or mechanism of action of TAF. The proliferation, formation and integrity of vascular endothelium are also involved in normal host processes: wound healing, morphogenetic tissue rearrangements, as well as the prevention of various non-neoplastic diseases resulting from blood vessel wall injury depend on angiogenesis factors⁶. A convenient method to study angiogenesis and endothelial repair is with an *in vitro* endothelial cell system, but the lack of uniform, reliable cultured endothelial cell lines has delayed progress in this area. Most studies⁷⁻⁹ (with some exceptions¹⁰) have used uncloned vascular cells which are often slow growing and heterogeneous in cell type. We have recently established a cloned adult bovine aortic endothelial (ABAE) cell line which has maintained its endothelial properties for over 1 yr in culture, provided that fibroblast growth factor (FGF) is present in

the growth medium¹¹. We report here the mitogenic effects of several untransformed and transformed cell lines on cloned ABAE cells and non-vascular endothelial cell controls.

Endothelial cell mitogenesis was monitored by seeding ABAE or control non-vascular adult bovine corneal endothelial (ABCE) cells on to γ -irradiated feeder layers of test cell cultures and measuring the proliferation of the unirradiated endothelial cells in the absence of FGF. All cells were cultured in Dulbecco modified Eagle's medium (DMEM) (ref. 12) with 10% calf serum. When vascular ABAE cells were seeded on to γ -irradiated feeder layers of MSV-transformed BALB/c 3T3 (MSV3T3) or BALB/c 3T3 (clone A-31) cells, substantial proliferation of ABAE cells occurred within two days (Fig. 1a). By 12 days ABAE cells grew to almost 100 times the density of ABAE cells grown in DMEM with 10% calf serum (Figs 1a and 2). MSV3T3 cells were almost as effective as FGF (100 ng ml⁻¹) and more effective than BALB/c 3T3 cells in promoting growth of ABAE cells, while NIH Swiss 3T3 and ABAE feeder cells had little effect on ABAE cell mitogenesis (Fig. 1a). When non-vascular ABCE cells were substituted

for ABAE cells, none of the cell lines tested stimulated ABCE cell proliferation, although ABCE cells do respond to FGF (Figs 1b and 2). Several other γ -irradiated untransformed cell lines were tested for their ability to promote the growth of ABAE cells. Although some of these cell lines caused slight increases in ABAE cell growth (15–41% over controls), none of the lines was judged to be significantly mitogenic to ABAE cells (Table 1). Thus far, every transformed cell line tested has stimulated ABAE but not ABCE cell proliferation (C.R.B. and G.L.N., to be published).

The proliferative effects of γ -irradiated BALB/c 3T3 and MSV3T3 cells on ABAE cells were due to substances released by the feeder cells and were not due to artefacts of γ -irradiation. This was demonstrated by experiments where the effects of conditioned medium from unirradiated feeder cells on ABAE cell proliferation were measured. Conditioned medium from BALB/c 3T3 and MSV3T3 cells stimulated growth of ABAE cells approximately 20 and 30 times, respectively, over ABAE cell growth in DMEM with 10% calf serum (Fig. 3). Although this was less than the effect of γ -irradiated BALB/c 3T3 or MSV3T3 cells on ABAE cell proliferation, it indicates that the former cell lines release an ABAE growth promoting factor(s) into the medium. Conditioned medium from ABAE cells was slightly more effective than γ -irradiated ABAE cells in promoting proliferation of the same cells but was much less effective than conditioned medium from BALB/c 3T3 or MSV3T3 cells. Conditioned medium from unirradiated BALB/c 3T3 or MSV3T3 did not stimulate proliferation of non-vascular ABCE cells (data not shown). These effects were not due to γ irradiation, because conditioned medium from γ -irradiated cells had the same effect as conditioned medium from non-irradiated cells, and mitomycin c-treated cells produced similar prolifera-

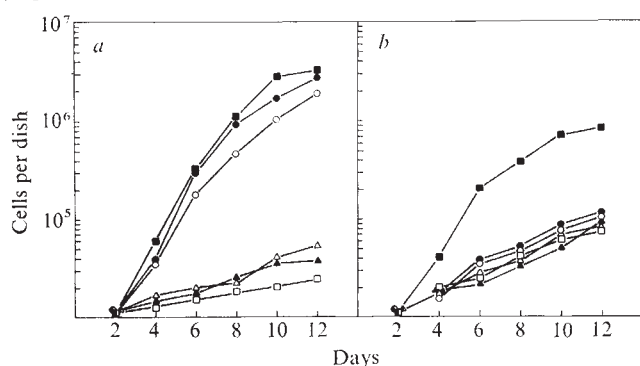
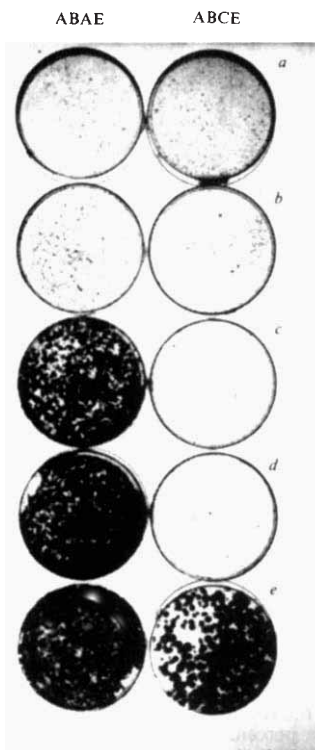


Fig. 1 Growth of adult bovine endothelial cells on γ -irradiated cells. *a*, Aortic endothelial cells; *b*, corneal endothelial cells. The cloning, culturing and characterisation of adult bovine aortic¹¹ and corneal¹³ endothelial cells have been previously described. The aortic endothelial cells are routinely cultured in the presence of 100 ng ml⁻¹ of FGF, which was purified as described^{14,20}. BALB/c 3T3 cells (clone A-31) and MSV3T3 cells (murine sarcoma virus-transformed clone A-31 BALB/c 3T3 cells) were obtained from Dr S. Aaronson of the National Cancer Institute, Bethesda, MD. BALB/c 3T3 cells grew as a density-inhibited line and were non-tumorigenic when 10⁶ cells were injected subcutaneously into BALB/c mice, whereas MSV3T3 cells had lost density-inhibition of growth and formed large tumours when 10⁶ cells were injected subcutaneously into BALB/c mice. NIH Swiss 3T3 cells were obtained from Dr K. Jones of the University of California, San Diego. All cell lines were cultured at 37 °C on Falcon tissue culture dishes in Dulbecco modified Eagle's medium (DMEM)¹² supplemented with 10% calf serum (Gibco). The *in vitro* assay of endothelial cell proliferation using γ -irradiated cells was conducted as follows. Six dishes were seeded 10,000 test cells in DMEM with 10% calf serum, and after the cells had attached (4–12 h), the dishes were γ -irradiated with a total of 6,000 R from a ⁶⁰Co irradiator to block cell division. The medium was then removed and 5,000 endothelial cells were seeded in DMEM with 10% calf serum on top of the test cell feeder layer. The following controls were employed: dishes of irradiated test cells alone, endothelial cells grown on γ -irradiated endothelial cells, and endothelial cells alone grown in the presence and absence of FGF (100 ng ml⁻¹); FGF was added every other day. In some experiments, confluent dishes of test cells were irradiated as described, removed from the dishes by trypsinisation and then seeded at 10,000 cells per dish. This did not alter the results of any experiments. Every other day after the addition of endothelial cells the medium was replaced on all dishes, and duplicate cultures were trypsinised and counted with an electronic cell counter (Electrozone/Celloscope, Elmhurst, Illinois). The number of endothelial cells growing on test cell feeder layers was corrected for the number of cells on dishes of test cells alone. The experiments were conducted five times and representative results are shown. □, Endothelial cells alone (*a*, aortic; *b*, corneal); ■, FGF (100 ng ml⁻¹); ●, γ -irradiated MSV3T3 feeder layer; ○, γ -irradiated BALB/c 3T3 feeder layer; △, γ -irradiated NIH Swiss 3T3 feeder layer; ▲, γ -irradiated endothelial feeder layer (*a*, aortic; *b*, corneal).

Fig. 2 Stained dishes of adult bovine endothelial cells growing on γ -irradiated cells. The experiment was conducted as described in Fig. 1. After 12 days the medium was removed and cells were stained with Diff-Quick (Harleco, Gibbstown, NJ). Control dishes were endothelial cells grown alone in DMEM with 10% calf serum. ABAE, adult bovine aortic endothelial cells; ABCE, adult bovine corneal endothelial cells. *a*, Control; *b*, γ -irradiated NIH Swiss 3T3 feeder layer; *c*, γ -irradiated BALB/c 3T3 feeder layer; *d*, γ -irradiated MSV3T3 feeder layer; *e*, FGF (100 ng ml⁻¹).



tive effects on ABAE cells when compared with γ -irradiated cells (C.R.B. and G.L.N. to be published).

The cloned ABAE cells used in the experiments here have been well characterised as endothelial cells¹¹. They can be continuously passaged in tissue culture without loss of endothelial cell characteristics when grown with purified FGF, however, in the absence of FGF they form small colonies of vacuolated, irregularly-shaped cells^{11,20}. Our results indicate that vascular ABAE, but not non-vascular ABCE cells, are sensitive to growth factors released into the medium by certain cultured cells. The relationship between these 'factors' and FGF is unknown, although these substances do not stimulate proliferation of ABCE cells which respond to FGF. Recent studies have demonstrated the effects of conditioned medium from various tumour cell lines on primary cell cultures from human umbilical cord endothelium⁸ and also the mitogenic properties of tumour cell homogenates on cultured foetal bovine aortic endothelial cells⁹. In both cases, the endothelial cells were mixed populations of uncloned cells and could not be passaged continuously. The cloned ABAE cells¹¹ should prove useful for endothelial cell proliferation studies, although it is important to note that the control of cell growth *in vitro* may differ from control *in vivo*.

Our findings that untransformed BALB/c 3T3 (A-31), but not untransformed NIH Swiss 3T3, cells significantly stimulate ABAE but not ABCE, cell growth may be related to other observations concerning BALB/c 3T3 cells. When BALB/c 3T3 (A-31) cells adherent to glass²¹ or plastic²² are implanted subcutaneously into BALB/c mice, haemangioma-endotheliomas²¹ or vasoformative sarcomas²² are formed, and Boone has suggested, on the basis of these experiments, that BALB/c 3T3 (A-31) cells may be preneoplastic. These results, together with morphological studies on BALB/c 3T3 cells²³ show that BALB/c 3T3 cells are probably of endothelial rather than fibroblast origin. BALB/c 3T3 cells possess nerve growth factor²⁴ and TAF (ref. 5) activities, but it is unclear if the presence of these factors is related to a preneoplastic state. If BALB/c 3T3 cells are endothelial in origin, our results cannot be simply interpreted as a conditioning effect of one type of endothelial cell on another, because ABAE cells have little effect on ABAE cell proliferation (Fig. 1a).

The nature of the factor(s) released by BALB/c 3T3 and MSV3T3 cells in our experiments is unknown. It seems to be different from FGF, since FGF but not BALB/c 3T3 or MSV3T3 cells stimulated non-vascular ABCE cells to proliferate (Figs 1b and 2). As mentioned earlier, Klagsburn *et al.*⁵ have shown that extracts of BALB/c 3T3 cells induce vascularisation when placed on the chorioallantoic membrane of the chicken embryo, whereas BALB/c primary mouse embryo fibroblasts have no effect. We have likewise

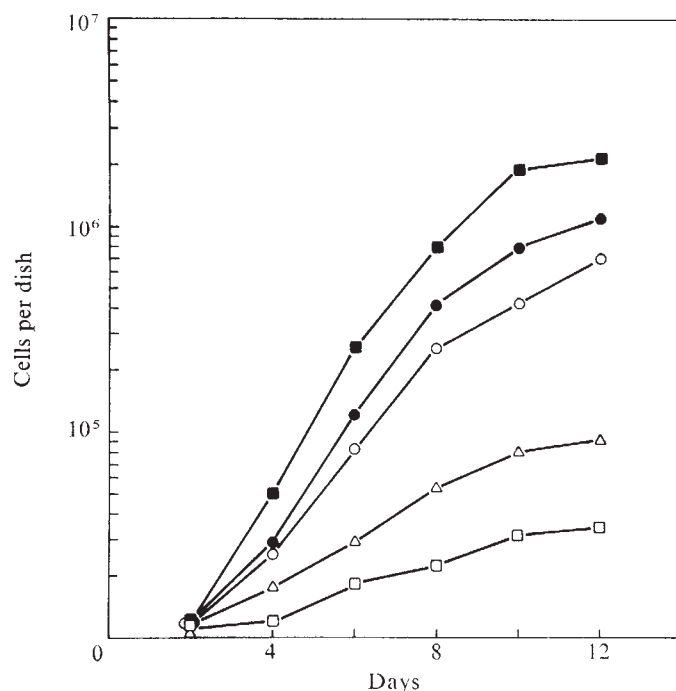


Fig. 3 Growth of adult bovine aortic endothelial cells in conditioned medium. For preparing conditioned medium of BALB/c 3T3 and MSV3T3 cells, 10^5 cells were seeded on to 10-cm dishes in 10 ml of DMEM with 10% calf serum. When the cells were subconfluent (about 4×10^6 cells per dish), the medium was removed, clarified by low-speed centrifugation and filtered through a 0.22- μ m filter, then frozen for storage. For preparing conditioned medium of adult bovine aortic endothelial cells, 10^6 cells were seeded as described, since these cells proliferate poorly when seeded at low densities in the absence of FGF. For growth rate studies, adult bovine endothelial cells were seeded at 5,000 cells per 6-cm dish in DMEM with 10% calf serum and after the cells had attached (4–12 h), the medium was replaced with fresh medium or the appropriate conditioned medium. Every other day after the addition of endothelial cells the medium was changed, and duplicate cultures were counted as described in Fig. 1.; FGF was added every other day. The experiments were conducted three times and representative results are shown. ■, FGF (100 ng ml^{-1}); □, DMEM with 10% calf serum; ●, MSV3T3 conditioned medium; ○, BALB/c 3T3 conditioned medium; △, aortic endothelial cell conditioned medium.

found that BALB/c 3T3 cells, but not BALB/c primary mouse embryo fibroblasts, will stimulate the proliferation of ABAE cells *in vitro*. It is unlikely that secreted proteolytic enzymes are responsible for the effects of BALB/c 3T3 cells on ABAE cells, since proteolytic enzymes such as thrombin have no effect on ABAE cell proliferation (D. Gospodarowicz, K. S. Brown, C. R. Birdwell and B. Zetter, to be published). Further study will be necessary to ascertain if the reported TAF activity of BALB/c 3T3 cells⁵ is responsible for the proliferation of ABAE cells *in vitro*. If this is the case, however, our results are the first to demonstrate that TAF is active *in vitro* as well as *in vivo*.

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Table 1 Effect of various cell lines on the proliferation of adult bovine aortic endothelial cells

Cell line*	% proliferation†
Bovine vascular smooth muscle cells	23
Bovine granulosa cells	18
Bovine corpus luteal cells	31
Bovine corneal epithelial cells	33
Bovine lens epithelial cells	28
Rat 31A ovarian cells	14
C57BL/6J primary embryo fibroblasts	29
BALB/c primary embryo fibroblasts	41

*The cloning and culturing of bovine smooth muscle¹¹, granulosa¹⁶, corpus luteal¹⁷, corneal epithelial¹⁸, lens epithelial¹⁹, and rat 31A ovary¹⁹ cells have been previously described. Mouse primary embryo fibroblasts were established by trypsinising 16 d-old embryos of C57BL/6J and BALB/c mice, respectively. All cell lines were cultured in DMEM with 10% calf serum (Gibco). The cell lines were tested for proliferative effect as described in Fig. 1, except that cultures were counted only on the twelfth day after the addition of endothelial cells.

†Refers to the percentage increase in the number of endothelial cells on γ -irradiated test cell feeder layers over the number of endothelial cells grown alone in DMEM with 10% calf serum.

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A T cell-replacing factor specific for histocompatibility antigens in mice

COLLABORATION between thymus-derived (T) and non-thymus-derived (B) lymphocytes in immune responses to many antigens has long been established¹. The actual mechanism of this collaboration, the role of macrophages, and whether it requires contact between the cells or is mediated by soluble factors is still under discussion. Although antigen-specific factors have been reported²⁻⁷, many T-cell replacing factors which have been described are not specific for the antigen used to prime T cells (see ref. 8 for review). Most of these non-specific factors have been obtained from cultures of allogeneic lymphocytes⁸⁻¹¹. We describe here a specific factor(s) obtained from lymphocytes stimulated *in vitro* by allogeneic cells. This factor replaces helper T-cells in an *in vivo* antibody response to the histocompatibility (H) antigens of the stimulating cells but not to other H antigens or to sheep erythrocytes (SRBC). This is the first description of a factor specific for H antigens which replaces T-cells in T-B collaboration.

Mixed spleen and lymph node cells from BALB/c (H-2^d) mice were stimulated in mixed lymphocyte culture (MLC) by C3H (H-2^k) cells. After 10 d in culture, the alloantigen 'primed' BALB/c lymphocytes were centrifuged and the supernatants were saved (culture supernatant). The cells (90-95% θ -positive T cells) were washed and resuspended in medium without serum at a concentration of 25×10^6 per ml and incubated at 37 °C for 4 h. The cell-free supernatant was collected after centrifugation (primed cell supernatant) and 0.2 ml was injected intravenously into BALB/c-nu (nude) mice immediately or after storage at -20 °C. After 2 weeks the nude mice were immunised against C3H and C57B1/6 (H-2^b) and tested for their ability to make an antibody response to H-2^k and H-2^b alloantigens. Some nudes were also immunised with SRBC. In this case, mice were immunised first with SRBC since prior immunisation with allogeneic cells has been found to increase the primary antibody response against SRBC (unpublished).

The results of one experiment are presented in Table 1. Nude mice injected with the primed cell supernatant made cytotoxic antibody against C3H cells which were used as stimulators in the MLC. Antibody activity was also detected against CBA cells, which share H-2^k with C3H. In contrast, no antibody activity was detected against C57B1/6 or C3H.SW, a strain congenic with C3H and bearing H-2^b. Nude mice injected with

supernatant from unprimed BALB/c cells or those not injected with supernatant did not make an antibody response. One of two nude mice injected with culture supernatant made a low-titred antibody response detected against CBA cells but not with any of the other test cells.

Nude mice given primed cell supernatant made antibody only after immunisation with cells (Table 2). One week after the injection of primed cell supernatant there was no detectable antibody against H-2^k or H-2^b. Following immunisation with cells from both strains, cytotoxic antibody was made to H-2^k only. The titre of the antibody was comparable with that made by normal BALB/c mice. After a second immunisation with C3H and C57B1/6 cells no antibody was made by the nude mice. In contrast, normal BALB/c mice produced higher antibody titres against both H-2 types when boosted.

Table 3 shows the antibody response of nude mice injected with primed cell supernatant or BALB/c lymphocytes and immunised with SRBC. While nude mice reconstituted with BALB/c cells made antibody to SRBC, the primed cell supernatant did not restore the ability of these mice to respond.

These results demonstrate that MLC-primed lymphocytes make a factor(s) which is capable of replacing T cells in the response to H antigens. The antibody response was specific for the H antigens present on the stimulator cell used in the MLC. Thus, factor from BALB/c cells primed against C3H enabled nude mice immunised with cells from C57B1/6 (H-2^b) and C3H (H-2^k) to make antibody which reacted with H-2^k bearing cells but not with H-2^b. The absence of antibody activity to C3H.SW is important since this demonstrates that the antibody responses of nude mice given the primed cell supernatant were restricted to the H-2 region. We do not yet

Table 1 Alloantibody responses of BALB/c-nu mice

Nude mice injected with*	Antibody response to†			
	C3H (H-2 ^k)	C57B1/6 (H-2 ^b)	CBA (H-2 ^k)	C3H.SW (H-2 ^b)
—	—‡	—	—	—
—	—	—	—	—
Primed cell supernatant	128 64 128 128	— — — —	128 64 128 128	— — — —
Unprimed BALB/c supernatant	—	—	—	—
Culture supernatant	—	—	4	—

*The primed cell supernatant was a cell-free supernatant prepared from MLC-primed BALB/c lymphocytes. 40×10^6 BALB/c cells (spleen and lymph node) were stimulated in MLC against 80×10^6 C3H cells (irradiated with 3,300 rad X ray) in 16 ml culture medium in flasks (Falcon 3024). The culture medium was RPMI 1640 made up to 5% with human serum, buffered with sodium bicarbonate (2 g l^{-1}) and HEPES (25 mM), and supplemented with L-glutamine (2 mM), L-arginine (0.6 mM), L-asparagine (0.3 mM), penicillin (100 IU ml^{-1}) and streptomycin ($100 \text{ } \mu\text{g ml}^{-1}$)²¹. After 10 d, the primed cells were washed and resuspended at 25×10^6 per ml in RPMI 1640 without serum and cultured at 37 °C for 4 h. The primed cell supernatant was recovered after centrifugation and used immediately or frozen before use. Unprimed BALB/c supernatant was a cell-free supernatant from freshly isolated, BALB/c lymphocytes (pooled spleen and lymph node) prepared in the same way as the primed cell supernatant. Culture supernatant designates the cell-free culture medium from the MLC. 0.2 ml of primed cell supernatant and cell supernatant from unprimed BALB/c lymphocytes were injected intravenously into nude mice; nude mice injected with culture supernatant were given 0.4 ml.

†Maximal antibody activity was measured on day 10 after immunisation intraperitoneally with 10^7 C3H and 10^7 C57B1/6 spleen cells by complement-dependent cytotoxicity using a two-stage method and absorbed guinea pig serum as a source of complement²¹. Responses are given as one/highest dilution at which antibody activity was detected. Each row represents responses of an individual mouse.

‡No cytotoxic antibody detected at a serum dilution of 1/4, the lowest dilution tested.

Table 2 Primary and secondary antibody responses of BALB/c mice or BALB/c-nu mice injected with primed cell supernatant

Mice*	Antibody response†					
	Pre-immunisation		Primary response		Secondary response	
	C57B1/6	C3H	C57B1/6	C3H	C57B1/6	C3H
BALB/c	—	—	32	32	128	256
	—	—	32	64	32	128
	—	—	32	64	128	256
	—	—	32	32	128	256
BALB/c-nu	—	—	—	128	—	—
+ Primed cell supernatant	—	—	—	64	—	—

*Nude mice were injected with 0.2 ml primed cell supernatant 14 days before immunisation by intraperitoneal injection with 10^7 C57 and 10^7 C3H cells. Each row represents the responses of an individual mouse.

†Given as one/highest dilution of serum giving detectable cytotoxic activity. Pre-immune sera from nude mice were tested on day 7 after injection of primed cell supernatant. The largest primary antibody response was measured between days 7 and 10 after immunisation in BALB/c mice and on day 10 in nude mice. Mice were re-immunised 14 d after the initial immunisation and the secondary response was measured 7 d later.

know the determinants within the H-2 complex against which the antibodies react, but K and/or D determinants are presumably involved since these antisera lysed 100% of lymph node cells.

The lack of T cell-dependent responses to SRBC in mice receiving the primed cell supernatant is of interest since a number of studies have demonstrated that alloantigen-stimulated lymphocytes make nonspecific factors which substitute for T cells in an *in vitro* response to SRBC (refs 8–11). But these factors were normally obtained early in the allogeneic reaction and tested *in vitro*. In our experiments the factor was obtained after 10 d in MLC when the proliferative response was ended and most of the responding (blast) lymphocytes had reverted to small cells. This suggests that, while lymphocytes responding in MLC may release nonspecific factors, elaboration of specific factors is either of a longer duration or requires a longer interval between antigenic stimulation and production of specific factor.

Factor with strong activity was recovered in the primed cell supernatants but not in the culture supernatants. The factor may have been present in the culture supernatants but at too low a concentration to be effective. Alternatively, serum components or other substances released by stimulated lymphocytes could have had an antagonistic effect on the activity of the factor.

Table 3 Antibody response of BALB/c-nu mice to SRBC

	Anti-SRBC haemagglutinating antibody†	
	Primary	Secondary
Untreated controls (2 experiments)	—	—
Nude mice injected with* Primed cell supernatant (4 experiments)	—	—
Culture supernatant (2 experiments)	—	—
BALB/c lymphocytes	3	5
	3	8
	5	8

*Primed cell supernatant (0.2 ml) and culture supernatant (0.4 ml) were injected; 10^7 BALB/c lymphocytes (pooled spleen and lymph node) were injected into control animals.

†Measured as $\log_2$ haemagglutinating activity by 2-mercaptoethanol (2-ME) treated sera, except for the secondary response of the mice injected with supernatant. 2-ME was used because non-reconstituted nudes sometimes make a 2-ME sensitive primary antibody response against SRBC. Antibody responses were measured 7 d after immunisation intraperitoneally with 10^8 SRBC.

The failure to find a secondary response (Table 2) in nude mice injected with the primed cell supernatant argues against a direct effect on precursor T cells known to be present in nude mice¹². Thus, the factor probably acts directly on specific B cells or has an indirect effect on B cells through macrophages. Conflicting evidence has been presented in the literature concerning the cell type to which antigen-specific factors are directed. Munro and Taussig¹³ found that B cells have an acceptor site for antigen-specific factors, suggesting an effect on B cells, whereas Feldmann^{14,15} has clearly demonstrated the importance of macrophages in his system.

In our system the nude mice failed to make a detectable antibody response unless challenged with allogeneic cells. Furthermore, the mice were immunised 2 weeks after injection of the primed cell supernatant, demonstrating that the activity of the factor is of long duration. This suggests that antigenic stimulation of specific B cells may not necessarily have to be temporally related to the T-cell help. Such a long term effect of the factor could be due to one of two mechanisms. Either the factor binds directly to specific B cells or to macrophages and is not degraded before antigenic challenge, or the factor gives a signal to the relevant B cells which commits these cells to making antibody on contact with antigen. In the latter case, the signal could result from direct binding to the B cells or after processing by macrophages.

Plate¹⁶ has described a factor which is obtained from MLC activated lymphocytes. This factor substitutes for helper T cells in the generation of cytotoxic T lymphocytes against H antigens. It is not known whether the same T-cell population acts as helpers in antibody production and in the generation of cytotoxic T-cells. But since the specificity of cytotoxic T cells and antibody to major H determinants may be similar^{17–20}, these systems obviously offer a unique opportunity for dissecting the nature of cell-cell interactions in the immune response.

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Magnitude of memory to the major histocompatibility complex

ALTHOUGH the phenomenon of memory is central to the adoptive immune response to antigenic stimulation, the magnitude of the memory effect in graft rejection has never been quantified. We have directly compared the potency

of naive and memory cells in two quantitative adoptive transfer assays, one measuring graft rejection and the other graft-versus-host activity. While the well known lack of a significant factor of immunisation in the latter was confirmed, memory cells showed a dramatic, long term increase in their potency to reject heart grafts.

Memory of prior exposure to antigens of the major histocompatibility complex (MHC) was shown many years ago to be both long-lived and specific¹. Long term memory to these antigens involves a surprisingly small quantitative change in immune responsiveness measured by conventional allo-immune assays, however. Naive animals rapidly reject MHC incompatible grafts and rejection of these grafts is not markedly accelerated by prior sensitisation². The factor of immunisation (FI) in graft-versus-host (GVH) assays is also negligible or absent^{3,4}. *In vitro*, although there is an acceleration in the kinetics of the response in mixed lymphocyte reactions, memory cells show no quantitative increase in proliferative capacity⁵. In all other immune responses (including those to weak alloantigens), long term memory is associated with a significant quantitative increase in the response^{2,3}.

It is possible that the failure to demonstrate memory to strong alloantigens reflects a deficiency in the assay systems used rather than the absence of a specific quantitative change in immune responsiveness in the lymphocytes of a host sensitised to these antigens. Evidence suggesting that this is so derives from adoptive transfer experiments. Sensitised cells differ markedly from naive cells in their capacity to terminate tolerance⁶ and to reject long-surviving grafts in immunologically privileged sites⁷. In both cases the response measured is primarily an effector response and not a proliferative one.

We have used a quantitative adoptive allograft assay to compare directly the potency of naive and sensitised cells in effecting the rejection of heterotopic heart grafts in irradiated rats. The same two cell populations were also compared in a quantitative GVH assay to detect any change in proliferative capacity resulting from long term sensitisation. Naive rats of the PVG (Ag-B5), DA (Ag-B4) and Lewis (Ag-B1) strains mutually rejected heterotopic adult heart grafts in 6–8 d (Fig. 1*a*). Previously sensitised animals of these strains showed the trivial acceleration of graft rejection which is expected in respect of major alloantigens (Fig. 1*b*). But, when naive and sensitised cells were compared in an adoptive assay rather than in the whole animal a dramatic increase in potency of sensitised cells was demonstrated. Rats irradiated with 750 rad did not reject Ag-B incompatible hearts for at least 40 d (Fig. 1*h*). Adoptive transfer of syngeneic lymphoid cells to irradiated recipients restored their capacity to reject heart grafts and the degree of restoration was related to the number of cells in the restorative inoculum (Fig. 1*d–f*). The minimum number of naive thoracic duct lymphocytes (TDL) or lymph node cells (LNC) which would restore graft rejection to first-set time was 500×10^6 (Fig. 1*d*). But, if the restorative inoculum was obtained from PVG donors bearing long term memory to DA alloantigens as few as 5 to 20×10^6 TDL or LNC caused rejection of DA hearts in first-set time (Fig. 1*j, k*). This represents an increase in potency of sensitised cells of 25–100-fold. The increase in potency is specific: PVG cells with memory for DA alloantigen behaved as naive cells in respect to Lewis heart grafts (Fig. 1*g, l*). Aliquots of the same cells which showed this dramatic increase in potency in respect of heart graft rejection showed no significant factor of immunisation in GVH responses (Fig. 2).

These results show that prior exposure to antigens of the MHC causes a dramatic and specific increase in potency of the cells responsible for allograft rejection but no increase in the proliferative capacity of the cells responsible for the GVH reaction. This suggests that the subpopulation of T

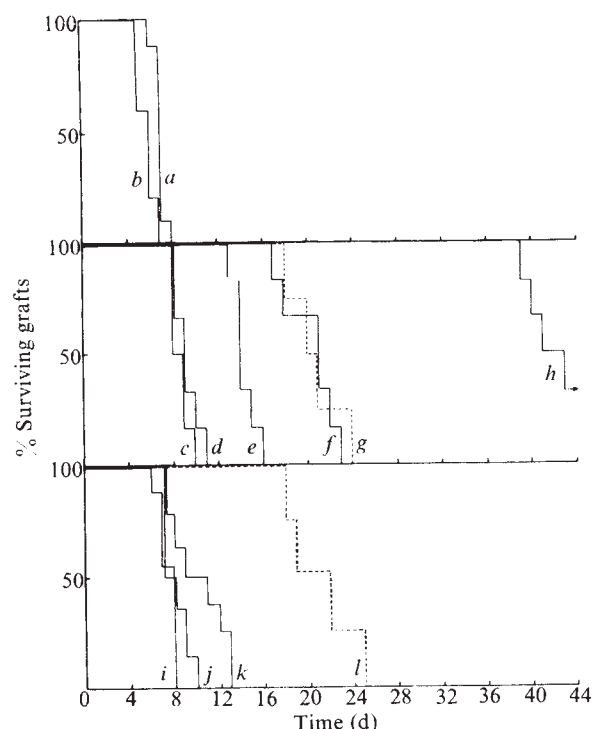


Fig. 1 Life tables of graft survival in PVG rats grafted with DA hearts (solid line) or Lewis hearts (broken line). The aorta and pulmonary arteries of the adult heart grafts were anastomosed to the abdominal aorta and inferior vena cava of the recipient using a modification of the technique of Ono and Lindsey¹⁶. Rejection was confirmed by loss of electrocardiogram activity. The PVG rats sensitised to DA alloantigens had rejected two sequential DA skin grafts, 6–12 weeks before use. Lines *a* and *b* represent rejection times of DA grafts in normal and sensitised recipients respectively. There is a very small difference in first and second-set rejection times of grafts between Ag-B different strains. *c–h* Represent rejection times of grafts in PVG rats given 750 rad on the morning of grafting. Irradiation was from a ^{60}Co source in conditions of maximum back scatter. Within 24 h of grafting groups of these recipients were injected intravenously with graduated doses of LNC from naive PVG rats. Doses of LNC given: *c*, 10^6 ; *d*, 5×10^6 ; *e*, 2×10^6 ; *f, g*, 5×10^7 . Graft survival was measured from the day of injection with LNC. Irradiated rats given no LNC (*h*) do not reject grafts for 40 d. The minimum number of LNC which will restore first-set rejection was 5×10^6 . With smaller numbers of LNC the acceleration of rejection is dose related. *i–l* Represent rejection of grafts in irradiated PVG rats injected with LNC from PVG rats sensitised to DA alloantigens. Doses of sensitised LNC given: *i, l*, 5×10^7 ; *j*, 2×10^7 ; *k*, 5×10^6 . Sensitised LNC at a dose of 2×10^7 always restores first-set rejection and at a dose of 5×10^6 restored half the rats to first-set. First-set rejection can be restored to irradiated rats with 25–100-fold fewer sensitised LNC than naive LNC. Specificity of the increased potency of sensitised cells was confirmed by showing that 5×10^7 LNC from rats sensitised to DA (*l*) restored Lewis graft rejection to the same tempo as the same number of naive LNC (*g*).

lymphocytes which proliferates in a GVH response and that which mediates tissue damage not only respond to different antigenic determinants⁸ and carry distinct cell surface markers⁹ but might also respond in quite a different manner to prior sensitisation. The successful demonstration *in vivo* of the dramatic and specific increase in allograft reactivity which occurs in the lymphocyte pool of rats which have been sensitised against antigens of the MHC depends on the use of an adoptive assay. A combination of two factors makes it impossible to demonstrate the extent of this change in the intact rat. It is well established that naive animals have large numbers of antigen sensitive cells responsive to strong alloantigens^{10,11}. The experiments reported here showed that there is a physiological limit to the speed with which detectable cell-mediated tissue damage can be effected. If less than 500×10^6 normal cells were given to an irradiated host, rejection time was directly

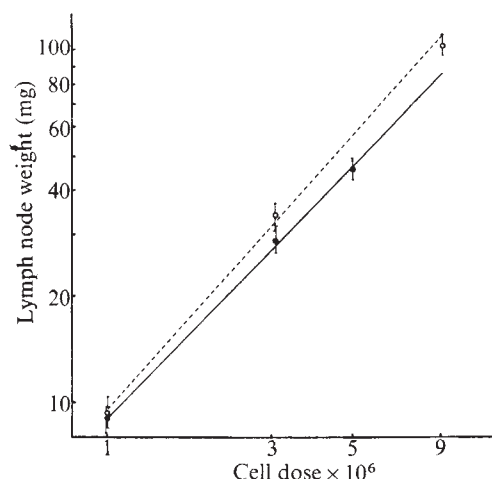


Fig. 2 Comparison of the GVH activity of LNC from normal and sensitised donors using the popliteal lymph node weight assay¹⁷. Varying numbers of LNC from PVG donors were injected into the right foot pad of groups of (DA $\times$ PVG) F_1 recipients matched for age and sex. Log lymph node weight (LNW) is plotted against log cell dose. Each point represents the arithmetic mean of at least 10 LNW. Vertical bars represent the s.e. The calculated regression lines are shown. ●, Response to PVG cells from normal donors (slope 1.0, correlation coefficient 0.94, s.d. of y 0.34). ○, Response to PVG cells from donors sensitised to DA alloantigen (slope 1.1, correlation coefficient 0.93, s.d. of y 0.41). The factor of immunisation at the lowest cell dose was 1.1 and the highest cell dose 1.3.

related to the cell dose (Fig. 1e, f). But, once this number was exceeded the response was not accelerated by increasing the number of adoptively transferred cells (Fig. 1c). 500×10^6 lymphocytes is only a fraction of the total lymphocyte complement of the intact rat¹². Thus the quantitative increase in cell potency of 25–100-fold which occurs after sensitisation to antigens of the MHC is not detectable in the whole animal because it is already redundant in allo-antigen sensitive cells. If, however, lymphocytes are adoptively transferred in limiting dilutions to an animal whose own cells have been destroyed by irradiation, the dramatic quantitative change in the lymphocyte pool of sensitised rats becomes obvious.

It has been shown that both GVH responses^{13,14} and skin graft rejection¹⁵ are mediated by pure populations of T cells. The most economical interpretation of our findings is that the clone of memory T cells which mediates graft rejection is functionally expanded 25–100-fold by prior immunisation. Failure of GVH assays to reflect this clonal expansion may indicate that the cells responsible for second set graft rejection are 'end' cells incapable of further proliferation.

Final proof that the increase in potency seen in the cells responsible for graft rejection is determined by antigens of the MHC and not minor histocompatibility antigens awaits confirmation in congenic strains. Preliminary experiments indicate, however, that the potency of lymphocyte populations sensitised against multiple minor histocompatibility antigens is more than an order of magnitude lower than that of cell populations sensitised against antigens of the MHC.

This dramatic effect of sensitisation on subsequent graft rejection has obvious relevance to clinical transplantation as well as to the fundamental biology of alloimmune responses. Investigations are at present in progress to determine the lifespan and distribution of the T cell responsible for these long term memory effects in the rat.

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Sex steroid hormone regulation of IgA and IgG in rat uterine secretions

SEX steroid hormones may be important in regulating both the systemic and secretory humoral immune system and on the basis of this hypothesis, we have carried out a series of experiments to clarify the action of the sex hormones on the rat uterine immune system. We report here that the stage of the oestrous cycle and the administration of physiological amounts of oestradiol to castrate rats markedly influences the accumulation of immunoglobulin A (IgA) and G (IgG) in uterine secretions. IgG is maximal at pro-oestrus but barely detectable at oestrus, dioestrus, and following ovariectomy. IgA is highest at pro-oestrus, remains elevated at oestrus, but drops to low levels at dioestrus and following ovariectomy. Of the steroids tested only oestradiol stimulated the appearance of IgA and IgG. These findings indicate that oestradiol is important in influencing the presence of both immunoglobulins in the uterus.

Our findings are of particular interest in the light of the fact that the use of the sex hormones to prevent conception and to reduce the discomfort of menopause has increased the incidence of a number of disorders in women^{1–3}, including alterations to the systemic immune system. Immunity to viral infections such as chicken pox, herpes simplex and rubella has been shown to be diminished among users of oral contraceptives⁴, and women taking oestrogen have been estimated to have a 1.49- to 1.81-fold greater risk of infection than those not taking it. Similar results have been obtained with the administration of sex steroids to rats and mice^{7,8}. Reports about the alteration in the local immune system in the female reproductive tract caused by sex steroids are somewhat conflicting. Although immune responsiveness and resistance to genital tract infection vary through the reproductive cycle (refs 9, 10 and 20), levels of IgA and IgG in cervical secretions have been reported by some to remain constant⁹ and by others^{11,12} to vary during the menstrual cycle. Furthermore, in the absence of apparent infection, oral contraceptives are reported to have altered antibodies in genital tract secretions, but whereas increased levels of immunoglobulins were reported with the use of combination oral contraceptives⁹, sharp decreases were observed after the use of the sequential pill^{11,12}.

Adult female virgin Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington) maintained in constant-temperature rooms with 12-h intervals of light and darkness were used in this study. Vaginal smears were made daily and females were selected at given stages of the

oestrous cycle after at least two consecutive normal 4-d cycles were observed. Ovariectomies were performed 10–15 d before each experiment. Following decapitation of the rats, uteri were perfused with sterile saline through the descending aorta to remove all blood from the vascular beds. Intact uteri were then removed and washed in 2–3 saline baths to eliminate adherent debris. A 26-gauge needle attached to a 1.0-ml tuberculin syringe containing 0.1 ml sterile saline was passed through the uterine wall at the ovarian end. As the saline was introduced into the lumen, a second syringe, with its needle inserted through the cervical musculature, was moved in synchrony to collect the uterine flushing. This procedure was repeated with the second uterine horn using the fluid recovered from the first. The fluid was centrifuged at 15,000g for 5 min, after which the supernatant was lyophilised and stored at -20°C until assayed. Blood was collected immediately after decapitation and was allowed to clot for 30 min at room temperature. Following centrifugation at 15,000g for 5 min, serum was removed and frozen at -20°C until assayed.

IgA and IgG were measured by radioimmunoassay. Rat IgG was purchased from Miles Laboratories; rabbit anti-rat heavy chain IgG antibody was a gift from Dr P. K. Chung (Miles Laboratories, Elkhart, Indiana); IgA-rich serum from rats with IR22 immunocytomas (IgA class) and goat anti-rat IgA (antiserum) prepared against this immunocytoma serum were gifts from Dr H. Bazin (Université Catholique de Louvain, Belgium). Anti-IgG and anti-IgA were coupled to cyanogen bromide-activated microcrystalline cellulose (Sigma, Sigma Chemicals, St Louis)¹³. The antigen components of the radioimmunoassay (rat IgG and rat IgA immunocytoma serum) were radio-labelled with ^{125}I using lactoperoxidase-bound Sepharose 4B (ref. 14). Uterine fluids were reconstituted with 50 μl of distilled water just before use and serum samples were diluted 200-fold before assay.

Figure 1a shows the levels of IgG in the uterine fluid of rats at various stages in the oestrous cycle and following castration. At pro-oestrus, the time in rats when plasma oestradiol is known to be highest¹⁵, IgG levels were significantly higher (10–15-fold) than those of dioestrus, oestrus, and after castration. No significant differences were measured in serum IgG concentrations (Fig. 1b). The

Fig. 1 Immunoglobulin G (a, b) and A (c, d) levels in uterine secretions (a, c) and serum (b, d) of adult female rats. Values represent the mean $\pm$ s.e.m. of 5–8 rats per group. IgG values are expressed as μg per uterus; IgA results as immunocytoma serum (IS) units, where 1.0 IS unit is defined as a concentration of 1 mg of lyophilised immunocytoma serum ml^{-1} distilled water. Values of IgG and IgA at pro-oestrus and IgA at oestrus are significantly different from those measured during dioestrus and following castration ($P \leq 0.01$). No significant differences were measured in serum samples.

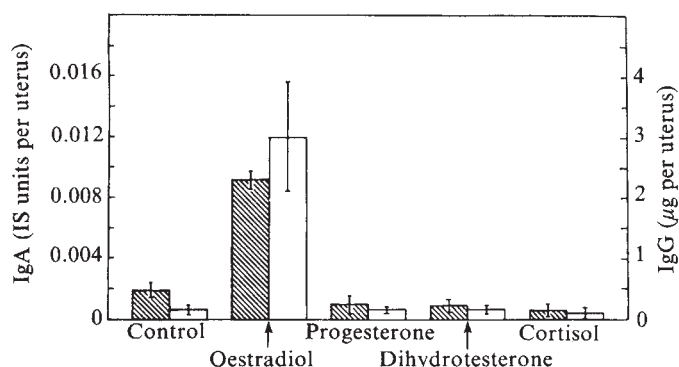
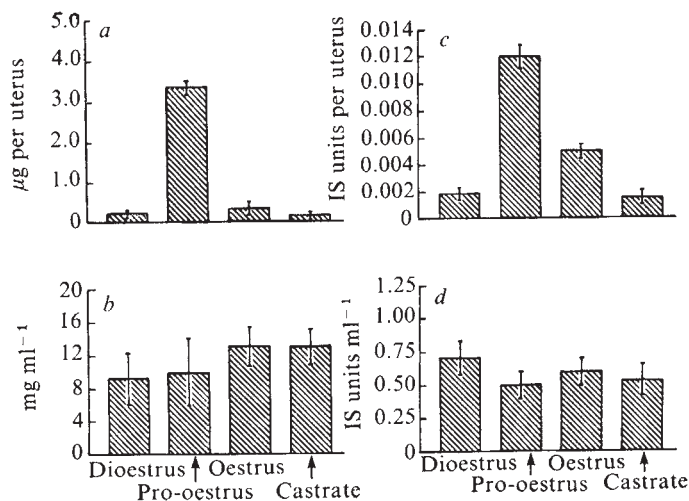


Fig. 2 IgA and IgG in uterine secretions of ovariectomised rats after three daily injections of steroids. Rats were injected with 0.1 ml of ethyl laurate containing either oestradiol (1.0 μg), progesterone (2,000 μg), dihydrotestosterone (1,000 μg) or cortisol (1,000 μg). Control groups received only ethyl laurate. Values represent the mean $\pm$ s.e.m. of 5 rats per group. The value for the oestradiol group was significantly higher ($P < 0.01$) than the values of the other groups. Hatched columns, IgA; plain columns, IgG.

corresponding mean values of IgA are shown in Fig. 1c. As observed with IgG, IgA is highest at pro-oestrus, but also remains partially elevated at oestrus. Serum IgA levels in these animals show no significant variations (Fig. 1d).

Oestradiol, progesterone, cortisol and dihydrotestosterone were administered at concentrations known to elicit physiological responses in their respective target tissues. Time-course studies (not shown) indicated that the antibody response, although detectable at 1 and 2 d, was maximal after 3 d of repeated (daily) injections of steroid. Oestradiol administered daily to castrate rats elevated both uterine IgG and IgA (Fig. 2). Similar results were observed when oestradiol was administered to hypophysectomised rats. In contrast, however, progesterone, cortisol and dihydrotestosterone had no effect.

These results show that uterine IgG and IgA are under oestrogen control and differ from serum levels in the same animals. The data do not enable us to conclude whether the changes observed result from selective transport or *de novo* synthesis of antibodies in this organ. It is known that there are cells in the genital tract mucosa that produce both IgA and IgG. Immunofluorescent labelling of antibodies has led to the identification of subepithelial IgA and IgG plasma cells of the cervix and uterine endometrium^{16,17}. In other studies, segments of uterine, cervical and vaginal tissues of the rabbit incorporate radioactive amino acids into IgA and IgG when incubated *in vitro* for 24 h (ref. 18). Similar techniques have also been used to demonstrate that these immunoglobulins are synthesised by the human cervix and vagina¹⁹. Further studies must be undertaken to determine which mechanism, transport or synthesis, is responsible for the oestrogen-influenced immunoglobulin accumulation in uterine secretions.

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Chemical structure of attachment sites for viruses on human erythrocytes

ENCEPHALOMYOCARDITIS (EMC) virus attaches to erythrocytes from a variety of species through terminal sialic acid residues of the cell surface membrane (M. A. Angel and A.T.H.B., in preparation). Glycophorin, the major sialoglycoprotein of human erythrocytes has been identified as a receptor for influenza virus and some lectins¹. In this report we present evidence that glycophorin is a human erythrocyte receptor for EMC virus also, and we have tentatively identified the location of a binding site for both EMC and influenza virus on glycophorin.

Glycophorin was prepared by the method of Marchesi and Andrews¹ from human type 0 erythrocytes, the cells used throughout these investigations. Electrophoresis of the glycophorin on 7.5% polyacrylamide gels in the presence of sodium dodecyl sulphate² gave a pattern (not shown) similar to that reported by Jackson *et al.*³—one major and a few minor components, possibly aggregates of glycophorin, were present, as revealed by staining either with Coomassie brilliant blue or with periodic acid–Schiff reagent.

Glycophorin was recognised as a receptor for influenza virus originally by its ability to inhibit virus haemagglutination^{1,3}. We found that both EMC and influenza virus haemagglutination were severely inhibited to about the same extent by glycophorin (Table 1). Influenza virus haemagglutination is inhibited by sialoglycoproteins from many sources⁶. To determine whether or not the inhibitory action of glycophorin

Table 1 Effect of glycoproteins on agglutination of human erythrocytes by EMC and influenza viruses

Sialoglycoprotein	Protein concentration* (µg per well)	Haemagglutination titres (% of untreated control)	
		EMC virus	Influenza virus
Human glycophorin	3.7	3	6
Bovine mucin	32.8	100	0.2
Human serum glycoprotein	43.8	100	100

Haemagglutination inhibition tests were performed using a microtitre system (Cooke Engineering) and, as diluent throughout, PBS–G–G, which comprised equal parts of 4.5% (w/v) glucose and PBS containing gelatin to 0.5% (w/v). Serial twofold dilutions of EMC or influenza virus, grown and purified as described previously^{2,4}, were made in 25-µl volumes and to each haemagglutination tray well was added either 25 µl of the sialoglycoprotein preparation or 25 µl of PBS–G–G in controls. After keeping the mixtures at 4 °C for 1 h, 50 µl of 0.1% (v/v) human erythrocytes were added to each well. The trays were maintained at least 5 h and usually overnight at 4 °C before haemagglutination titres were recorded. The reduction in haemagglutination caused by the sialoglycoprotein treatment is expressed as a percentage of the titre in untreated virus controls.

*Protein concentrations measured by the procedure of Lowry *et al.*⁵.

Table 2 Inhibition of EMC virus attachment by glycophorin

(µg protein per sample)	% Attachment
0	75
0.4	35
0.8	23
8.0	0

To 10 µl of ³H-amino acid-labelled EMC virus, prepared as already described² was added 10 µl of a glycophorin preparation (10 µl PBS in controls) and after 1 h at ambient temperature, 1 ml of 0.1% (v/v) human erythrocytes in PBS–G–G, (PBS–G–G alone in controls) was added. The samples were maintained a further 1 h at ambient temperature before centrifuging at 3000g for 5 min to collect the supernatant fluid. The erythrocytes (or deposits, where present in the absence of erythrocytes) were washed once in PBS–G–G before resuspending in 1 ml of PBS–G–G. After addition of 0.5 ml 30% H₂O₂ to all samples, to bleach the haemoglobin, the radioactive contents of erythrocytes (or deposits), supernatant fluid and wash, were measured in a Beckman LS 250 scintillation spectrometer. % Attachment represents the c.p.m. in the erythrocyte sample compared with the total c.p.m. recovered.

was a property of sialoglycoproteins in general, bovine submaxillary gland mucin (Worthington) and human serum glycoprotein (Mann Research) were tested, but neither inhibited EMC virus haemagglutination even at about 10 times the protein concentration at which glycophorin was effective (Table 1). In contrast, influenza virus haemagglutination was inhibited by mucin although not by human serum glycoprotein (Table 1).

To test whether glycophorin inhibited attachment in addition to the subsequent agglutination step, ³H-amino acid-labelled EMC virus, was mixed with various concentrations of glycophorin before measuring the ability of the virus to bind to erythrocytes. In the absence of glycophorin, 70–80% of the recovered radioactivity was bound by the erythrocytes whereas in the presence of glycophorin (0.8 µg glycophorin protein⁵ per sample) this was reduced to 23% (Table 2). At higher glycophorin levels (8.0 µg protein per sample), attachment was virtually eliminated as revealed by recovery of background levels only of radioactivity in the erythrocytes.

Pretreatment of human erythrocytes with neuraminidase reduced EMC virus attachment to about 15% of that of untreated controls and usually abolished haemagglutination by EMC or influenza virus (Table 3). Neuraminidase, previously heated at 100 °C for 10 min, had no such effect, showing that this was an efficient means for inactivating the enzyme. If glycophorin is the molecule to which EMC virus binds on the erythrocyte surface, neuraminidase treatment of glycophorin should abolish its ability to inhibit attachment of EMC virus. This was found to be the case; glycophorin (0.8 µg protein per sample) pretreated with neuraminidase (0.5 units per sample) had almost no inhibitory effect against EMC virus, attachment being 86% of that in the absence of glycophorin (not shown). The loss of inhibitory properties did not result from the heating procedure required to destroy the neuraminidase since glycophorin, heated but not treated with enzyme, reduced EMC virus attachment to 8% and thus showed enhanced inhibitory properties compared with unheated glycophorin at the same concentration (Table 2).

To determine how glycophorin interfered with virus attachment, 25 µl of ³H-labelled EMC virus was mixed with 25 µl of glycophorin, bovine submaxillary gland mucin or human serum glycoprotein (each containing 100 µg protein), or with phosphate-buffered saline (PBS), and after incubating at ambient temperature for 60 min, each sample was examined by sucrose gradient sedimentation. The samples incubated with mucin, human serum glycoprotein or PBS all showed a single peak of radioactivity at a position characteristic of EMC virus, whereas most of the radioactivity in the glycophorin sample sedimented to the bottom of the tube (Fig. 1). A similar result was obtained when each sialoglycoprotein sample was adjusted to the same sialic acid content (12 µg sialic acid per sample as determined by the thiobarbituric acid procedure of Warren⁷) rather than the

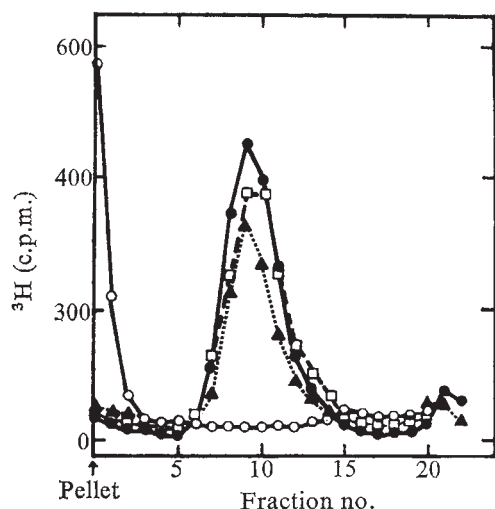


Fig. 1 Rate of zonal sedimentation of ^3H -labelled EMC virus incubated for 60 min at ambient temperature with glycophorin ($\circ$), mucin ($\square$), human serum glycoprotein ($\bullet$) or PBS ($\blacktriangle$) before analysis on 10–30% sucrose gradients containing 0.1 M NaCl and 0.02 M phosphate buffer, pH 8.0 in a Spinco SW 50.1 rotor at 50,000 r.p.m. for 30 min. Direction of sedimentation is from right to left.

same protein content. These results suggest that glycophorin, unlike mucin and human serum glycoprotein, attaches to EMC virus-forming aggregates and in so doing inhibits attachment of virus to cells. However, the aggregates are apparently not sufficiently large to sediment with erythrocytes in the conditions of low-speed centrifugation used to measure attachment of virus to cells.

The work of Marchesi and colleagues^{3,8} suggests that trypsin treatment of intact human erythrocytes releases from glycophorin amino acids 1–39, numbered from the N terminus, and leaves projecting from the cell surface a short sequence containing about 25 amino acids beyond which the glycophorin is embedded in the cell surface membrane. We found that trypsin-treated erythrocytes consistently gave haemagglutination titres equal to, or up to threefold dilutions higher for both EMC and influenza virus and bound about the same amount of ^3H -labelled EMC virus, compared with controls incubated in the absence of trypsin; typical results are given in Table 3. This suggests that both EMC and influenza virus can attach to the 25 amino acid segment of glycophorin remaining exposed on the cell surface after trypsin treatment. The results do not exclude the possibility, suggested for influenza virus from the work of Marchesi and colleagues³, that virus attachment sites are also present in the 39 amino acids released by trypsin.

The ability of trypsin treated cells to bind ^3H -labelled EMC virus or to be agglutinated by either EMC or influenza virus

was greatly reduced if the cells were subjected to further incubation with neuraminidase (Table 3). A similar reduction in haemagglutination titre was observed for cells treated with neuraminidase before trypsin (Table 3). These results suggest that sialic acid residues in the fragment remaining with the cells after trypsin treatment are involved in attachment of both EMC and influenza virus to human erythrocytes. Only three amino acids in this fragment have sialic acid attached: serine at positions 44 and 47 and threonine in position 50 (ref. 8). We conclude, therefore, that one or more of these three sialic acid groupings on glycophorin is involved in the binding of EMC or influenza virus by human erythrocytes.

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Synthesis of low molecular weight inhibitor of protein synthesis with enzyme from interferon-treated cells

PROTEIN synthesis in cell-free systems from mouse L cells pretreated with the antiviral agent interferon¹ shows an enhanced sensitivity to inhibition by double-stranded RNA (dsRNA) (refs 2–4). The inhibition of protein synthesis is dependent on incubation with ATP as well as dsRNA and we and others have reported a dsRNA-dependent protein kinase activity(s) in extracts from interferon-treated cells^{5–8}. The possible involvement of a viral dsRNA-mediated inhibition of protein synthesis in the sequence of events following virus infection in intact, interferon-treated cells has been discussed previously^{2,4}. The situation with extracts from interferon-treated cells^{5–8} is reminiscent of that in rabbit reticulocyte lysates in which phosphorylation of an initiation factor by a protein kinase has been implicated in the inhibition of protein synthesis in a variety of conditions including the presence of dsRNA^{9–12}. In the interferon-treated L-cell system, however, in addition to the kinase, a heat-stable, low molecular weight inhibitor (LMW inhibitor) of protein synthesis is formed on incubation with ATP and dsRNA (ref. 6). It remained possible from our previous work⁶, that the LMW inhibitor might be a small phosphorylated peptide product of the dsRNA-dependent kinase. We show here that this is not the case. In addition, we show that the enzyme responsible for the synthesis of the LMW inhibitor will bind to a column of dsRNA attached to a solid support. It can be eluted in high salt but is relatively unstable in this form. We have used the highly-purified enzyme in its stable column-bound state to synthesise and radioactively label the LMW inhibitor. This synthesis, the partial purification of the inhibitor and its activity in the inhibition of protein

Table 3 Agglutination of enzyme-treated erythrocytes by EMC and influenza viruses

Enzyme treatment	% of untreated controls		Attachment of ^3H -EMC virus
	EMC virus	Influenza virus	
Neuraminidase	0	0.3	15.0
Trypsin	200	400	98.0
Trypsin then neuraminidase	2.3	0.3	17.6
Neuraminidase then trypsin	3.1	1.0	ND*

Preparations of 0.1% (v/v) human erythrocytes were incubated at 37 °C for 30 min with 1 mg ml⁻¹ trypsin (Worthington) or with 50 units per ml *Vibrio cholerae* neuraminidase (Calbiochem). The enzymes were removed by washing before applying the second enzyme treatment and/or using for haemagglutination or virus attachment studies as described in Table 2.

*ND, Not determined.

synthesis in cell-free systems from L cells and rabbit reticulocytes, are reported here. A more detailed characterisation of the inhibitor is described in the accompanying paper¹³.

The enzyme(s) responsible for the synthesis of the LMW inhibitor (inhibitory enzyme) binds to dsRNA, so chromatography on columns of poly(I):poly(C) bound to Sepharose (poly IC-Sepharose) (refs 14, 15) were used to purify this enzyme(s) and the dsRNA-dependent protein kinase(s). (Preliminary results indicate that the two activities can be separated, A.G.H., unpublished). On passage of a post-ribosomal supernatant fraction from interferon-treated cells (interferon cell sap) through such a column, the inhibitory enzyme and kinase activities were retained. The inhibitory enzyme was recovered

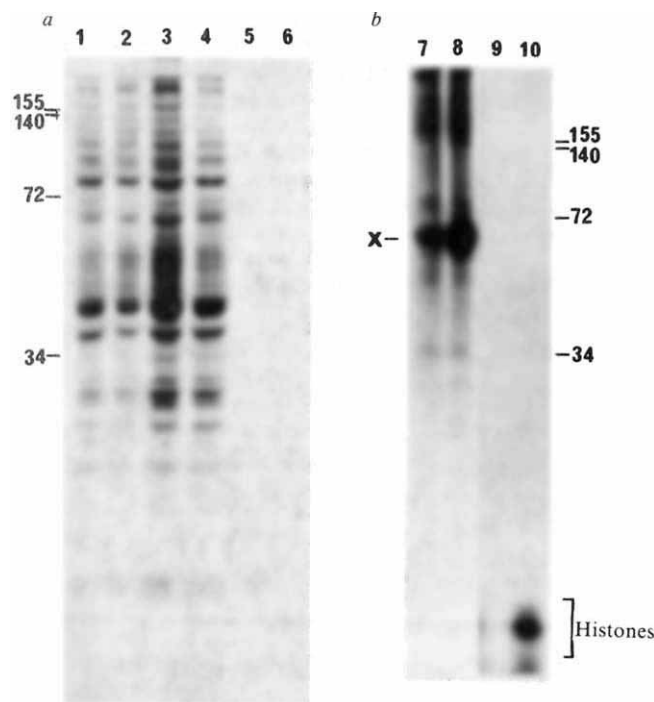


Fig. 1 Chromatography of interferon and control cell saps on poly IC-Sepharose: electrophoretic analysis on polyacrylamide (10%) slab gels containing sodium dodecyl sulphate (SDS) (refs 16, 17). Interferon or control cell saps (50 μ l) were passed through columns of poly IC-Sepharose (50 μ l) equilibrated with dialysis buffer containing glycerol (DBG: 10 mM HEPES, pH 7.5, 90 mM KCl, 1.5 mM magnesium acetate, 7 mM 2-mercaptoethanol and 20% glycerol, v/v). The columns were extensively washed with DBG and the starting material, that retained on the column and the initial pass-through, were analysed for protein and protein kinase activity. *a*, Gel stained for protein: control (1) and interferon (2) cell sap (2 μ l), control (3) and interferon (4) pass-through (equivalent to 4 μ l of cell sap). For protein retained on the column, the control (5) and interferon (6)-poly IC-Sepharose were heated (95 °C, 10 min) in sample buffer containing SDS (ref. 16), centrifuged and supernatant equivalent to 50 μ l of cell sap applied to the gel. *b*, Kinase activity: an autoradiograph of a stained, dried gel is shown. Control (7) and interferon (8) poly IC-Sepharose incubated 60 min at 30 °C with 0.15 mM γ -³²P-ATP (7.1 Ci mmol⁻¹, Radiochemical Centre, Amersham) in DBG containing 10 mM magnesium acetate. Phosphorylation of histone: control (9) and interferon (10)-poly IC-Sepharose samples were incubated (60 min, 30 °C) with 1 mM ATP. Calf thymus histone (type IIA, Sigma, 1 mg ml⁻¹) and γ -³²P-ATP (0.15 mM, as above) were added and incubation continued for 30 min. The poly IC-Sepharose samples (7-10) were heated (95 °C, 10 min) in sample buffer containing SDS and supernatants equivalent to 7 μ l of cell sap applied to the gel. The numbers to the left and right give the molecular weights in thousands of the major reovirus polypeptides λ 1, λ 2, μ 2 and σ 3 (ref. 18). The phosphorylated polypeptide band X corresponds to that previously observed on incubation of interferon cell sap with γ -³²P-ATP and dsRNA (Figs 1 and 4, ref. 6). Interferon treatment of mouse L cells with low doses (5 to 20 effective units per ml) of highly-purified mouse interferon ($\geq 5 \times 10^7$ units per mg protein), the preparation of cell extracts and of interferon and control cell saps were as described previously²⁻⁴.

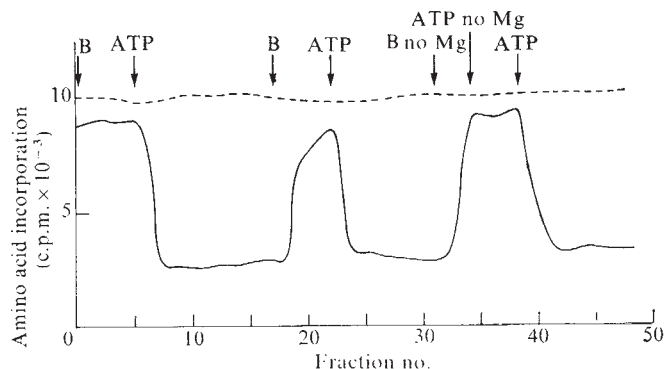


Fig. 2 Synthesis of the LMW inhibitor. Control (---) and interferon (—) poly IC-Sepharose columns (250 μ l) were prepared as in Fig. 1, washed extensively with DBG and incubated at 30 °C in the different conditions indicated (arrows). Abbreviations: B = DBG; ATP = DBG plus 1 mM ATP; B no Mg = DBG minus magnesium acetate; ATP no Mg = DBG plus 1 mM ATP minus magnesium acetate. For each set of conditions the columns were stopped and incubated for 20 min at 30 °C before collection of each 300- μ l fraction. Samples diluted 50-fold were assayed for their inhibitory activity on cell-free protein synthesis^{4-6,17}. The inhibitory activity of the samples was unaffected by heating at 95 °C for 5 min (data not shown). The ordinate gives the incorporation (c.p.m. per 10 μ l sample) of a ¹⁴C-amino acid mixture into hot trichloroacetic acid-insoluble polypeptide in response to encephalomyocarditis virus RNA (EMC RNA) in an L cell-free system^{4-6,17}.

on elution with high salt (1 M KCl) at a purification of more than 1,000-fold (a minimum figure is given since the amount of protein in the eluate was too low to be measured). The 1 M KCl eluate contained all of the recoverable inhibitory enzyme activity ($\geq 25\%$), assayed either by the formation of the LMW inhibitor on incubation with ATP and dsRNA (refs 5, 6), or by its addition with dsRNA to an L cell-free system^{4,9}. No such activity was detected with corresponding control cell material.

Although it was not possible to detect protein on the interferon and control poly IC-Sepharose columns (prepared as in Fig. 1) by conventional techniques (Fig. 1a), when these columns were incubated with γ -³²P-ATP phosphorylated polypeptides were detected (Fig. 1b). The extent of phosphorylation of a polypeptide of approximately 60,000 in apparent molecular weight (band X, Fig. 1b) was the most striking difference between the interferon and control preparations. By analogy with the reticulocyte lysate system¹⁰, this phosphorylation is thought to represent autophosphorylation of the dsRNA-dependent protein kinase⁶. Bands Y and Z reported previously for interferon cell sap (Figs 1 and 4, ref. 6) were not observed here, presumably because the appropriate substrates were not retained on the column. The protein kinase activity(s) of the interferon and control preparations were further investigated by incubation of interferon and control poly IC-Sepharose which had been pre-incubated with unlabelled ATP, with additional histone and γ -³²P-ATP. In these conditions histone was phosphorylated with the interferon but not the control poly IC-Sepharose (Fig. 1b, channels 9 and 10).

The inhibitory enzyme bound to poly IC-Sepharose was active in the synthesis of the LMW inhibitor. The formation of the inhibitor was dependent on Mg²⁺ and could be controlled by the provision or withdrawal of ATP (Fig. 2). No inhibitor was formed with corresponding control material (Fig. 2). The requirement for ATP suggested that it might form an integral part of the LMW inhibitor, so an attempt was made to label the LMW inhibitor by incubation of the column-bound enzyme with ³H-ATP. In these conditions label (³H) was incorporated into material which co-purified with the LMW inhibitor on chromatography on DEAE-cellulose and Sephadex G-25 (Fig. 3). The behaviour of this column-synthesised inhibitor on the latter was, incidentally, identical to that previously described for the LMW inhibitor made by incubation of crude interferon cell sap with ATP and dsRNA, being retarded on the column but eluting before marker ATP (Fig. 3,

ref. 6). Further evidence that ATP forms an integral part of the inhibitory molecule is presented in the accompanying paper¹³.

As here we seemed to be dealing with an inhibitor of a type not previously characterised, it was of interest to see if it had activity in different cell-free systems. The LMW inhibitor, in fact, showed similar activity in the inhibition of endogenous or EMC RNA-directed protein synthesis in the rabbit reticulocyte lysate system, to that observed in the L cell-free system (Fig. 4). The kinetics of inhibition in both systems with the LMW inhibitor were similar to those observed with dsRNA or the haem-controlled repressor in the reticulocyte lysate^{9-12,19}. In each case there was a lag of 10–15 min before onset of the inhibition (Fig. 4). In view of this and the effectiveness of the LMW inhibitor at subnanomolar concentrations¹³, it remains possible that the LMW inhibitor is not directly inhibitory but that it, in turn, regulates the formation of an inhibitor(s) or subsequent inhibitory events.

We have shown here that the enzyme(s) responsible for the synthesis of the LMW inhibitor and a protein kinase(s) are retained on poly IC-Sepharose. The poly IC-Sepharose column, therefore, provides a possible basis for their separation, characterisation and the determination of their respective roles in

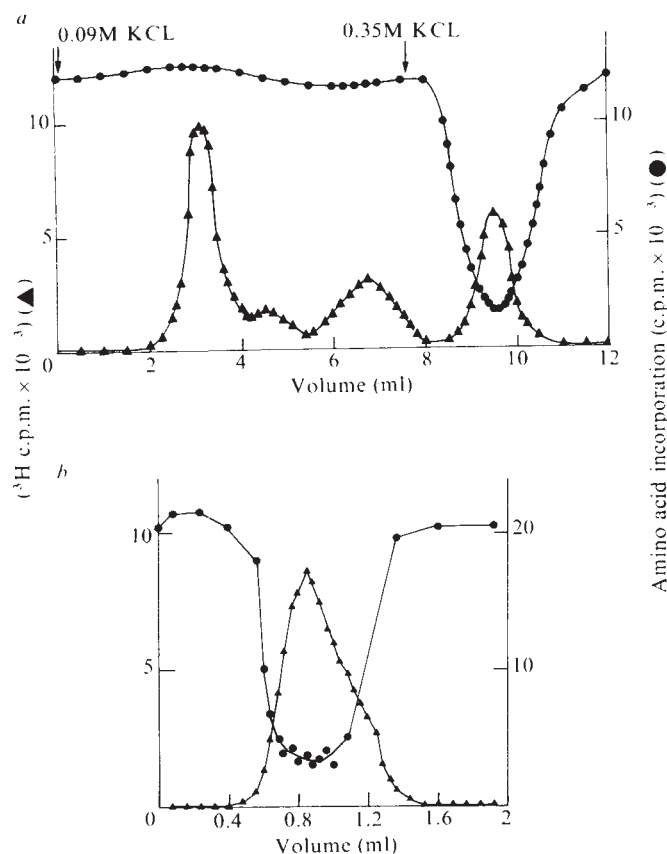


Fig. 3 Purification of the LMW inhibitor on DEAE-cellulose and Sephadex G-25. The LMW inhibitor was synthesised on a column of interferon-poly IC-Sepharose (Figs 1, 2) by incubation for 17 h at 30 °C in the presence of 1 mM ^3H -ATP (60 Ci mol⁻¹, Radiochemical Centre, Amersham) concentrated by acetone precipitation and applied (a) to a DEAE-cellulose column (0.3 $\times$ 3 cm) equilibrated in DBG. After extensive washing with DBG (containing 0.09 M KCl, arrowed), to remove residual ^3H -ATP and ^3H -labelled by-products lacking inhibitory activity, the LMW inhibitor was eluted at higher salt (0.35 M KCl). Peak fractions containing the LMW inhibitor were pooled, concentrated by acetone precipitation and chromatographed (b) on Sephadex G-25 (fine) as described previously⁶. In these conditions excluded material elutes between 0.3 and 0.5 ml and ATP between 0.9 and 1.5 ml (compare with Fig. 3, ref. 6). Samples (at a final dilution of 2,500-fold in a and 10,000-fold in b) were assayed for inhibitory activity on cell-free protein synthesis⁴⁻⁶. The right hand ordinates give the incorporation of ^{14}C -amino acids in an L cell-free system as in Fig. 2 (●). Those on the left show the radioactivity in each fraction (^3H c.p.m. per 5- μl sample) originating from the ^3H -ATP (▲).

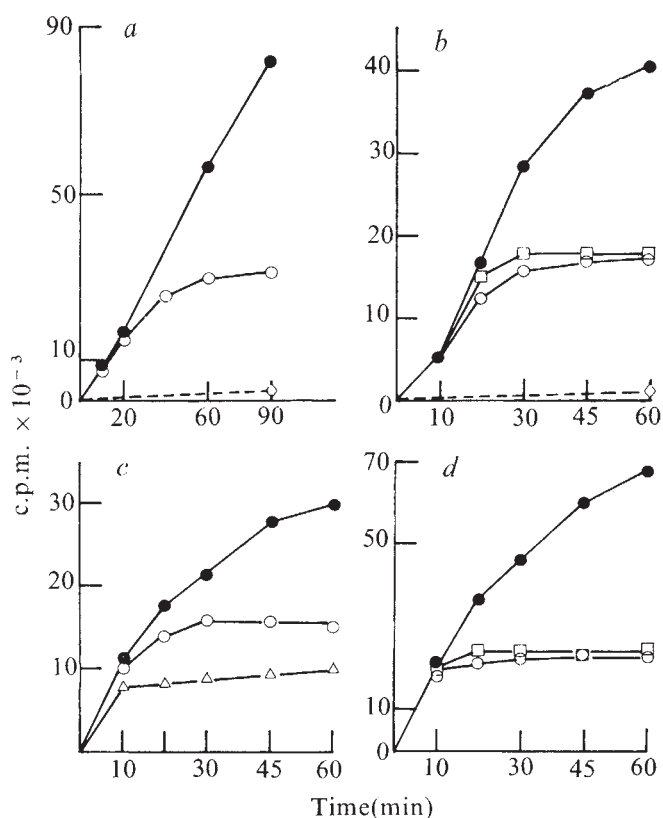


Fig. 4 Assay of the LMW inhibitor in cell-free systems from mouse L cells and rabbit reticulocytes: comparison with dsRNA and the haem-controlled repressor (HCR) (refs 5–8, 19). a, b, Translation of EMC RNA in the absence (●) or presence of the LMW inhibitor (○) or poly(I)-poly(C) (□) in cell-free systems using: a, preincubated and dialysed S10 from mouse L cells¹⁷. b, A micrococcal nuclease-treated rabbit reticulocyte lysate²⁰. There was negligible incorporation in the absence of EMC RNA (◇). c and d, Amino acid incorporation in the absence (●) or presence of the LMW inhibitor (○), poly(I)-poly(C) (□) or HCR (△) in an untreated rabbit reticulocyte lysate system in the absence (c) or presence (d) of EMC RNA (16 $\mu\text{g ml}^{-1}$). The ordinates give the incorporation (c.p.m. per 3 μl sample) of ^{35}S -methionine (200–500 Ci mmol⁻¹, Radiochemical Centre, Amersham) into hot trichloroacetic acid-insoluble polypeptide¹⁷. The same concentration (1 μl of a 300-fold dilution per 12.5 μl assay) of the same preparation of the LMW inhibitor purified on DEAE-cellulose was used in all cases. (Its inhibitory effect in the reticulocyte lysate was not due to the carryover of poly(I)-poly(C) from the Sepharose column. Any such material was removed by the DEAE-cellulose step⁶. In addition, similar results have been obtained with material further purified on Sephadex G-25 (Fig. 3b) and by paper electrophoresis¹³.) Poly(I)-poly(C) (Sigma) was added to a final concentration of 100 ng ml⁻¹. The HCR was prepared and used as before²¹. It and the rabbit reticulocyte lysates were gifts of M. J. Clemens.

the inhibitory process(s). Meanwhile the column technique has provided a simple, convenient method for the synthesis of the LMW inhibitor. The availability of radioactively-labelled inhibitor synthesised in this way has considerably facilitated its initial characterisation¹³ and should prove invaluable in the elucidation of its structure, mechanism of action and role in the inhibition of cell-free protein synthesis by dsRNA.

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Nature of inhibitor of cell-free protein synthesis formed in response to interferon and double-stranded RNA

PROTEIN synthesis in cell-free systems from mouse L-cells pre-treated with the antiviral agent interferon¹ shows an enhanced sensitivity to inhibition by double-stranded RNA (dsRNA) (refs 2–4). A dsRNA-dependent protein kinase^{5–7} and a heat-stable, low-molecular-weight inhibitor (LMW inhibitor) of protein synthesis which is formed in such systems on incubation with ATP and dsRNA (ref. 7), seem likely to be involved. We have shown⁸ that the LMW inhibitor can be conveniently synthesised using a highly-purified enzyme fraction from interferon-treated cells bound to dsRNA on an inert support. Here we report its partial characterisation.

Throughout this work the LMW inhibitor was assayed by its ability to inhibit the translation of encephalomyocarditis virus RNA (EMC RNA) in cell-free systems from mouse L-cells^{4,7}. It was synthesised in the presence of ³H-ATP or ATP labelled with ³²P in the α or γ position using an enzyme fraction bound to a poly(I):poly(C)-Sepharose column⁸ or, as previously, by incubation of crude cell sap from interferon-treated cells with ATP and dsRNA (refs 4, 7). After chromatography on DEAE-cellulose to remove residual ATP (ref. 8), the different inhibitor preparations were analysed by electrophoresis on paper at pH 3.5 (Fig. 1). The inhibitory activity eluted as a single peak (Fig. 1a) coincident with the major peaks of ³H, α - or γ -³²P (Fig. 1a–c). This was true for the intrinsic inhibitory activity of the radioactively-labelled, column-synthesised material or for the unlabelled inhibitor made in crude extracts. It seems, therefore, that the inhibitor synthesised using the column method is not an artefact of the column procedure, but is identical to that formed in response to dsRNA in cell-free systems from interferon-treated cells. Moreover it can be labelled with ³H-ATP or with α - or γ -³²P-ATP. The column-bound enzyme⁸ is extensively washed before use in the synthesis of the inhibitor. It seems unlikely, therefore, that any low molecular weight component other than ATP (as much as 20% of which is consumed in the formation of the inhibitor) is required in significant amounts for its synthesis.

It is possible to calculate the concentration of AMP in the purified LMW inhibitor preparations from the observed ³H or α -³²P counts and the known specific activity of the radioactive ATP used in its synthesis on the column⁸. In four different preparations the dilution of inhibitor required to produce a 50% inhibition of protein synthesis in the cell-free system corresponded to an AMP concentration of 0.3 to 1 nM (Fig. 2). Accepting the intrinsic variability of the cell-free assay and that the inhibitor preparations were not completely pure

(contamination $\leq 30\%$, I.M.K. and R.E.B., unpublished results), the correspondence between the four preparations, two labelled with ³H-ATP and two with α -³²P-ATP, is remarkably good. Accordingly these data, together with those presented in Fig. 1 and the accompanying paper⁸, strongly suggest that ATP is a substrate in the synthesis of the inhibitor and forms an integral part of the biologically active molecule. Further evidence for this and a partial characterisation of the inhibitor was provided by the experiments described in Fig. 3.

Inhibitor was synthesised in the presence of α -³²P-ATP, chromatographed on DEAE-cellulose⁸ and subjected to enzy-

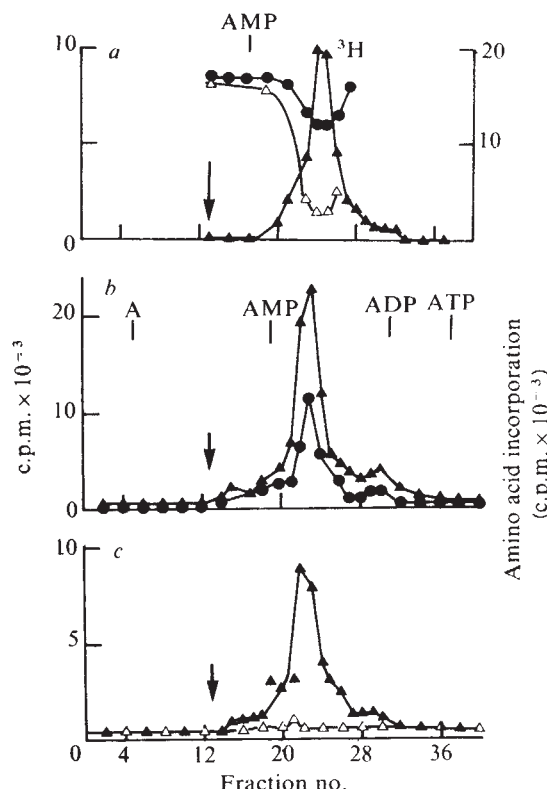
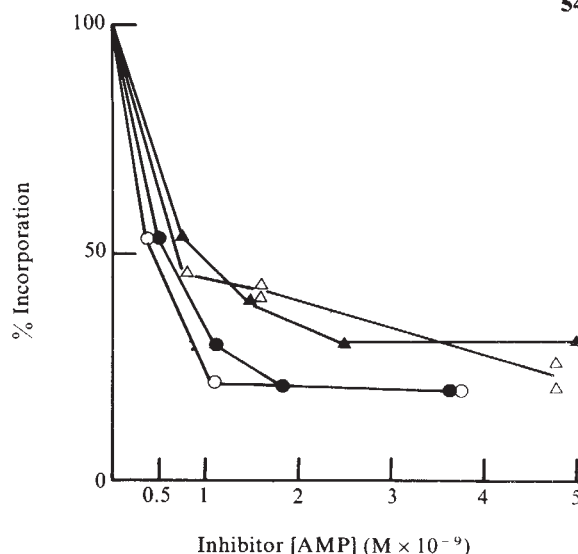


Fig. 1 Electrophoretic analysis of the LMW inhibitor. The LMW inhibitor was synthesised in the presence of ³H-ATP, α - or γ -³²P-ATP with an enzyme fraction bound to a column⁸, or by incubation of unlabelled ATP with cell sap from interferon-treated cells and dsRNA (refs 4, 7). After chromatography on DEAE-cellulose⁸, peak fractions were subjected to electrophoresis on Whatman 3MM paper in pyridine-acetic acid-water (1:10:989, v/v/v) pH 3.5 at 4,000 V for 45 min. The paper was dried, cut and 1-cm sections either eluted for assay of inhibitory activity in the cell-free system or counted for radioactivity. In each case the arrow indicates the origin with the cathode to the left. The position to which unlabelled A, AMP, ADP and ATP markers migrated is indicated. (In this system the inhibitor is easily distinguished from any residual ATP). *a*, Inhibitor synthesised in the presence of ³H-ATP (ref. 8) or as previously with cold ATP (refs 4, 7) were electrophoresed in parallel and analysed for radioactivity: ($\bullet$), ³H, left-hand ordinate) and for inhibitory activity in the cell-free system. The right-hand ordinate shows the incorporation of a ¹⁴C-amino acid mixture into hot trichloroacetic acid-insoluble polypeptide in response to EMC RNA in a cell-free system from control mouse L cells^{4,7} (c.p.m. per 10- μ l sample) assayed in the presence of eluted material at an overall dilution of 100 (Δ) or 10³-fold ($\bullet$). There was no inhibition with corresponding control material. *b*, Inhibitor preparations synthesised by the column method⁸ in the presence of $\bullet$, ³H-ATP and Δ , α -³²P-ATP electrophoresed in parallel. *c*, Distribution of ³²P in interferon (Δ) and corresponding control (Δ) preparations synthesised in the presence of γ -³²P-ATP. For the control, interferon cell sap was replaced by control cell sap in the synthetic procedure described in the accompanying paper⁸. Similar controls have yielded similar results with ³H-ATP and α -³²P-ATP. For inhibitor synthesised in the presence of ³H-ATP, α - or γ -³²P-ATP the intrinsic inhibitory activity migrated with the major peak of radioactivity (data not shown). The ³H-ATP (27 Ci mmol⁻¹) and α and γ -³²P-ATP (5 to 15 Ci mmol⁻¹) were obtained from the Radiochemical Centre, Amersham).

Fig. 2 Concentration of AMP in the inhibitor preparations for 50% inhibition of protein synthesis in the cell-free system. The LMW inhibitor was synthesised by the column method⁸ in the presence of 1 mM ATP in a total column volume of 0.15 to 0.9 ml. Radioactive ATP (as in Fig. 1) was added as follows: ³H-ATP to a final specific activity of 145 (○) and 120 (●) Ci mol⁻¹, α-³²P-ATP to a final specific activity of 18 (△) and 135 (▲) Ci mol⁻¹. After chromatography on DEAE-cellulose to remove residual ATP (ref. 8) samples containing ³H or ³²P were either dried on to glass fibre filters and counted in a Beckman scintillation counter using a toluene-based scintillation fluid with efficiencies of 30% and ≥ 90% respectively, or assayed at suitable dilution (1 μl of a 1,000–20,000-fold dilution per 12.5-μl assay) for inhibitory activity in the cell-free system. The concentration of AMP in the cell-free system assay (abscissa) was calculated from the known specific activity, the counts in the sample and the dilution used in the assay. Inhibitory activity in the cell-free system was assayed as described in Fig. 1: the ¹⁴C-amino acid incorporation for assays in the presence of different dilutions of the inhibitor is expressed as a percentage of the control (no inhibitor) value which varied from 8,000 to 20,000 c.p.m. per 10 μl sample in different experiments.



ApA (Fig. 3a), (Ap)₂A or (Ap)₃A included as internal markers. An identical spectrum of resistance and sensitivity to these agents has been obtained with inhibitor labelled with ³H-ATP or γ-³²P-ATP and with unlabelled inhibitor formed in crude interferon cell sap^{4,7}. (In the latter two cases sensitivity to T2 and U2 has not been tested.) Radioactivity (major spot, arrowed Fig. 3a) and inhibitory activity were lost in parallel on partial digestion with alkali or BAP. With γ-³²P-ATP-labelled inhibitor, digestion with BAP alone liberated all of the ³²P as inorganic phosphate (P_i) with corresponding loss of

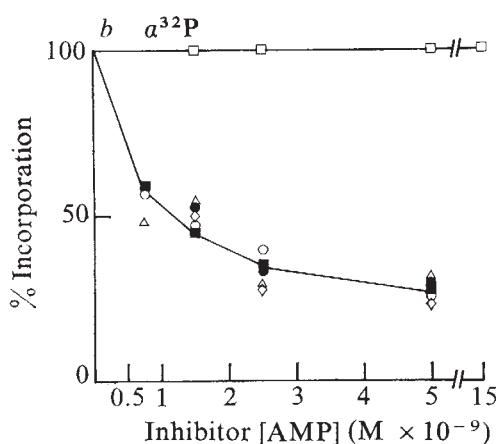
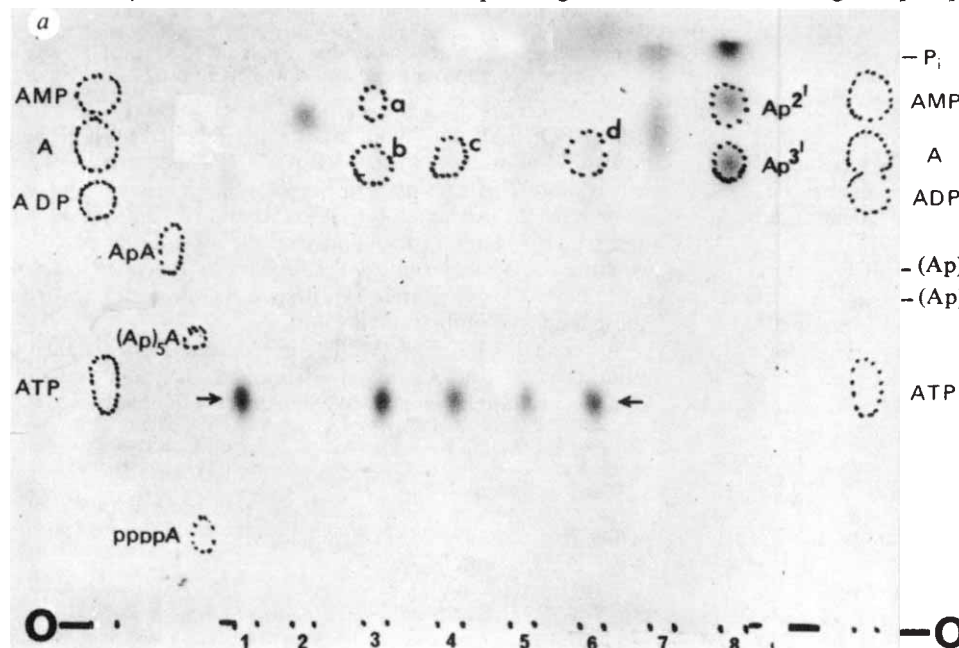


Fig. 3 Enzyme susceptibility of the LMW inhibitor. The LMW inhibitor was synthesised in the presence of α-³²P-ATP by the column method and chromatographed on DEAE-cellulose to remove residual ATP (ref. 8). It was subjected to enzymatic and alkali digestion followed either by *a*, chromatographic analysis in 0.75 M potassium phosphate, pH 3.4, on thin-layer plates of PEI cellulose⁹ (Machery Nagel, Polygram Cel 300 PEI, Camlab), or *b*, assay for inhibitory activity in the cell-free system as described in Figs 1 and 2. The ordinate and abscissa (*b*) were as described in Fig. 2. *a*, 1, Untreated inhibitor; 2–8, inhibitor after digestion with SVPD, P1, T2, micrococcal and U2 nucleases, BAP and BAP followed by alkali, respectively. *b*, ■, Untreated inhibitor; △, ○, ●, ◇, inhibitor after digestion with P1, T2, U2 and micrococcal nucleases, respectively; □, inhibitor after digestion with SVPD, BAP or BAP plus alkali. (Similar results were obtained for biological activity after digestion with alkali alone, data not shown.) Enzyme digestions were carried out in 5–20 μl reactions for 1–6 h at 37 °C in the conditions

described by Brownlee¹⁰ (for T2, U2 and micrococcal nucleases, SVPD and BAP) and Rose¹¹ (for P1). Alkali digestion was for 17 h in 0.3 M KOH. An autoradiograph of the dried plate is shown (*a*). In *a*, 3, 4 and 6 an approximately 100-fold excess of unlabelled ApA was added to the α-³²P-labelled inhibitor before digestion to provide a positive control showing enzyme activity. Spots *a*, *b*, *c* and *d* were detected under ultraviolet light and are the digestion products of the ApA—*a*, p(5')A; *b*, A; *c*, e, A and A(3')p. Digestion was without effect on the inhibitor (arrowed) here or in parallel experiments in which the same digestions were carried out in the absence of ApA (data not shown). The slight variation in the intensity of the inhibitor spot (arrowed) reflects variation in the amounts loaded. The optical density markers (dotted circles) A, AMP, ADP, ATP, Ap (mixture of 2' and 3'), A(3')p, ApA and pA were from Sigma and (Ap)₂A and (Ap)₃A from Boehringer. (Ap)₂A and micrococcal nuclease were from PL Biochemicals. The SVPD and P1 (from *Penicillium citrinum*), T2 (RNase T2 from *Aspergillus*)¹⁰ and U2 (RNase U2 from *Ustilago*)¹⁰ nucleases were gifts of Dr Beverley Griffin. BAP was 'BAPF' from Worthington.

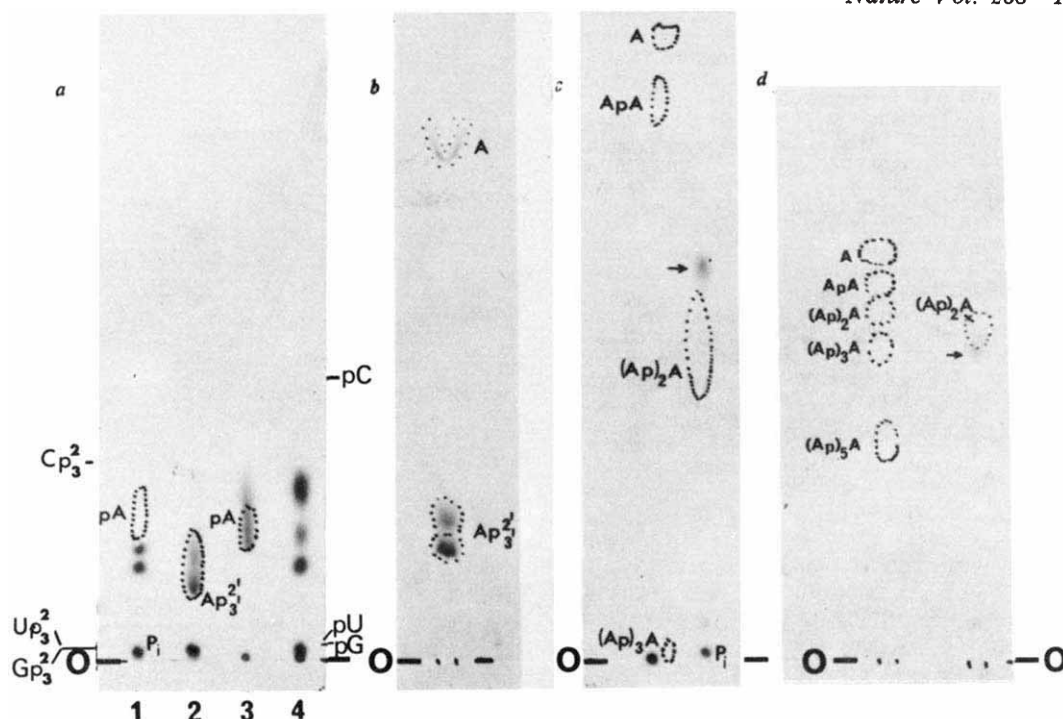


Fig. 4 Analysis of the products of SVPD, BAP and BAP followed by alkali digestion of the ^3H or $\alpha\text{-}^{32}\text{P}$ -labelled inhibitor. LMW inhibitor was prepared and subjected to enzymatic and alkali digestion as described in Fig. 3, before further chromatographic analysis of the digestion products, *a*, $\alpha\text{-}^{32}\text{P}$ -labelled inhibitor: 1 and 2, alkali digest of BAP-treated inhibitor with unlabelled pA or Ap (mix of 2' and 3'), respectively, as markers. 3, SVPD digest with unlabelled pA marker. 4, Mixture of alkali digest of BAP-treated inhibitor plus SVPD digest of untreated inhibitor. *b*, ^3H -labelled inhibitor: alkali digest of BAP-treated inhibitor. *c*, *d*, $\alpha\text{-}^{32}\text{P}$ -labelled inhibitor: BAP resistant core (arrowed). Thin-layer chromatography was in 1 M acetic acid on PEI cellulose¹² for *a*, *b* and *c*; for *d* it was in *n*-propanol-concentrated ammonia-water (55:10:35, v/v/v) on cellulose (Polygram Cel 300, Machery Nagel, Camlab, Cambridge, England)¹³. Optical density markers (dotted circles) were as in Fig. 3. Detection of radioactivity was by autoradiography in *a*, *c* and *d* and by fluorography¹⁴ in *b*.

biological activity. It seems reasonable to conclude that the major radioactive component (arrow Fig. 3*a*) is indeed the biologically active inhibitor. Accordingly, a more detailed analysis of its digestion products was undertaken to obtain further information concerning its structure.

Digestion of the $\alpha\text{-}^{32}\text{P}$ or ^3H -labelled inhibitor with SVPD yielded 5' AMP as the only major product (Fig. 3*a* track 2, and Fig. 4*a* track 3 for ^{32}P ; data for ^3H not presented). Sequential degradation with BAP and alkali yielded $^{32}\text{P}_i$, 2'-AMP and 3' AMP (in approximately equal amounts, Fig. 3*a* track 8 and Fig. 4*a* tracks 1 and 2) and ^3H -labelled adenosine, 2' AMP and 3' AMP (Fig. 4*b*). The BAP-resistant core of the molecule, however, is not simply ApApA. It migrates to a point intermediate between $(\text{Ap})_2\text{A}$ and $(\text{Ap})_3\text{A}$ on chromatography in a system which separates according to size (Fig. 4*d*) and migrates with neither of these markers when chromatographed on PEI cellulose with 0.75 M potassium phosphate pH 3.4 (Fig. 3*a*) or acetic acid (Fig. 4*c*) solvents. Moreover, even after removal of all BAP-sensitive phosphate the $\alpha\text{-}^{32}\text{P}$ or ^3H -labelled 'BAP core' of the inhibitor remains resistant to, for example, P1 and T2 nucleases in conditions of complete digestion of ApA or $(\text{Ap})_2\text{A}$ included in the same reaction mix (data not presented). The possible involvement of pA residues linked 2'-5' rather than 3'-5' has not been tested. Overall it seems unlikely from these results that the LMW inhibitor corresponds directly to the possible 'pleiotypic activator' A(5')pppp(5')A (ref. 15), or to the various analogues of the 5'-terminal cap structures of messenger RNA (ref. 16). These latter, for example, seem to be required at much higher concentration ($\geq 10 \mu\text{M}$) than the LMW inhibitor to be effective in the inhibition of cell-free protein synthesis¹⁷⁻²⁰.

From our previous work it remained possible that the heat-stable LMW inhibitor might be a small phosphorylated polypeptide product of the dsRNA-dependent protein kinase present in extracts from interferon-treated cells. This is not the case. Here we have shown that although the inhibitor is resistant to a variety of nucleases it is sensitive to digestion

with SVPD, BAP and alkali and can be labelled with ^3H and $\alpha\text{-}^{32}\text{P}$ -ATP as well as with $\gamma\text{-}^{32}\text{P}$ -ATP. It apparently contains two or more AMP residues in SVPD and alkali-labile linkage. Its precise nature remains to be determined, but the results suggest that it is a 'di- or oligonucleotide' of unusual or modified structure which is effective at subnanomolar concentrations in the inhibition of protein synthesis in cell-free systems from mouse L-cells or rabbit reticulocytes.

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Effects of sodium butyrate on synthesis of human chorionic gonadotrophin in trophoblastic and non-trophoblastic tumours

SOME human tumours and tumour-derived cell lines are capable of producing 'eutopic' peptide hormones and other proteins that are normally synthesised by the tissue from which the tumour originated. For example, choriocarcinoma cells, which are derived from cancer of the placental trophoblast, can synthesise human chorionic gonadotrophin (hCG) (refs 1, 2), and cultured pituitary adenomas can produce growth hormone (GH) (ref. 3). On the other hand, it is well established that a variety of malignant human tumours can produce 'ectopic' peptide hormones and other proteins which are not normally associated with the tissue of origin⁴⁻⁸. Ectopic synthesis of placental proteins, including the placental isoenzyme of alkaline phosphatase as well as the hormones hCG and human placental lactogen, may provide specific markers for neoplasm, since these proteins are not normally found in measurable amounts in the serum of men or of non-pregnant women⁹. Several human glycoprotein trophic hormones, including hCG, thyroid-stimulating hormone (TSH), luteinising hormone (LH),

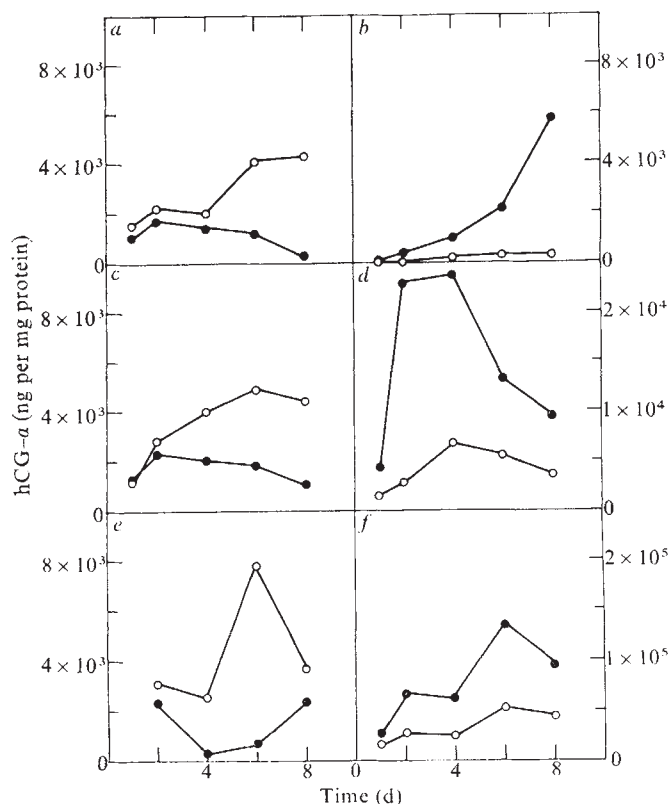


Fig. 1 Effect of sodium butyrate on hCG- α synthesis. *a*, JEG-3; *b*, HeLa CCL2; *c*, Reid; *d*, HeLa S₃; *e*, BeWo; *f*, ChaGo. Cultures were grown at 37 °C in 25-cm² flasks in 5 ml of α MEM (BeWo, ChaGo and HeLa cells) or Ham's F-12 (JEG-3 and Reid) supplemented with 10% foetal bovine serum. Media and serum were obtained from Flow Laboratories, Rockville, Maryland. Media were replaced every 2 d. hCG- α and hCG concentrations in the culture medium were measured by a specific double-antibody radioimmunoassay using hCG- α and hCG as reference standards¹⁵. For measurement of cellular protein content, cells were collected by removing the medium, washing the cells twice with saline, and scraping the cells off the plastic surface with a rubber policeman. The cells were collected by centrifugation and then frozen. The frozen pellet was resuspended in 0.3 ml buffered saline (0.9% NaCl in 0.01 M Tris-HCl, pH 7.4), and the cells were ruptured by sonication (Raytheon Magnetostrictive Oscillator, Model DF-101, Raytheon, Manchester, New Hampshire). The sonicates were centrifuged at 10,000g for 15 min. Total protein in the supernatant solutions was determined by the method of Lowry *et al.*¹⁶. $\circ$, Control; $\bullet$, 2 mM sodium butyrate.

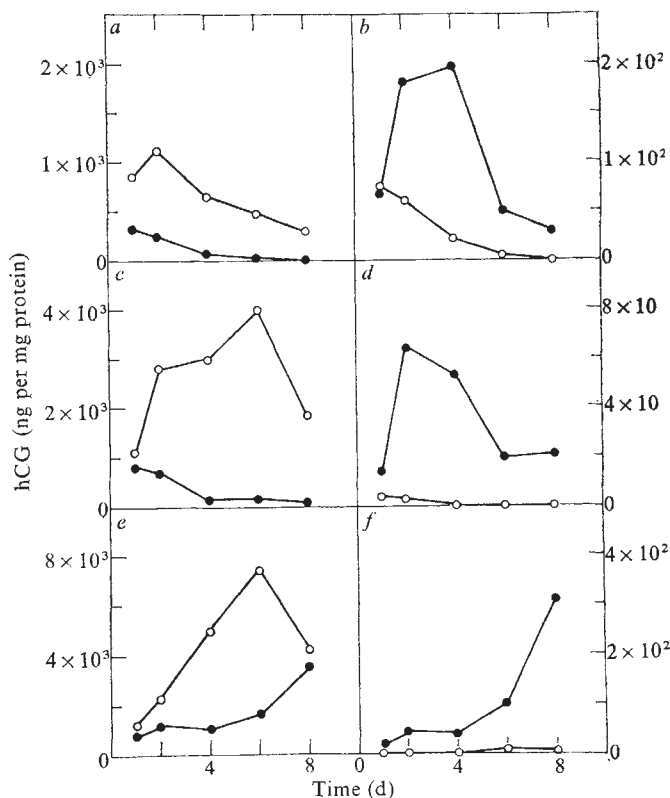
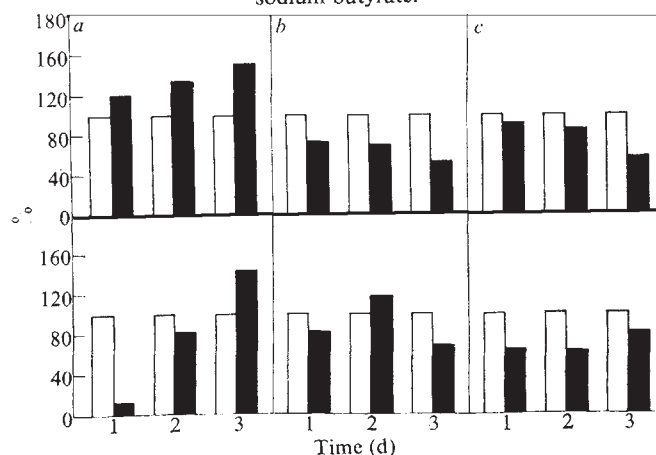


Fig. 2 Effect of sodium butyrate on hCG synthesis. *a-f* And conditions as in Fig. 1. $\circ$, Control; $\bullet$, 2 mM sodium butyrate.

and follicle-stimulating hormone (FSH), are composed of two non-identical subunits, α and β (refs 10-13). These four hormones have virtually identical α subunits; in contrast each has a unique β subunit, which is responsible for biological specificity¹². Complete hCG and free subunits are secreted by

Fig. 3 Effect of sodium butyrate on the rate of synthesis of cell DNA, RNA and protein. Monolayers of JEG-3 (top row) or HeLa S₃ cells (bottom row) were incubated with medium containing 2 mM sodium butyrate. At indicated times, cells were labelled with (a) methyl-³H-thymidine (5 μ Ci ml⁻¹), (b) ³H-uridine (5 μ Ci ml⁻¹), or (c) ³H-leucine (5 μ Ci ml⁻¹), for 1 h at 37 °C (radioisotopes were purchased from New England Nuclear, Boston, Massachusetts). After removal of the radioactive medium, the cultures were washed with phosphate-buffered saline, and the cells were lysed with 0.01 M Tris-HCl, pH 7.5, containing 0.6% SDS and 10 mM EDTA. Macromolecules in samples of the lysates were precipitated with an equal volume of 10% cold TCA. The precipitates were collected on GFC glass fibre filter disk (Reeve Angel Laboratory, Clifton, New Jersey) and washed extensively with 5% TCA. The filters were dried and the radioactivities were measured. Data are expressed as percentages of control values. Open bars, control; solid bars, sodium butyrate.



normal and neoplastic placenta^{1,2,14} and by many non-trophoblastic tumours^{15,16}. Since hCG is a eutopic hormone for trophoblastic tumours, but an ectopic hormone for non-trophoblastic tumours, it occurred to us that its synthesis by the two types of tumours might be regulated differently. We therefore compared the synthesis of hCG and hCG- α by three choriocarcinoma cell lines—BeWo (ref. 1), JEG-3 (ref. 2) and Reid (P. O. Kohler, unpublished observations)—with the synthesis by two non-trophoblastic tumour-derived cell lines—ChaGo and HeLa. (ChaGo was derived from an hCG-producing pulmonary carcinoma¹⁶, and HeLa from a carcinoma of the cervix¹⁷.) We also tested the effects of sodium butyrate on the synthesis, since this compound had been shown to induce hCG- α and hCG synthesis in certain HeLa cells¹⁸. We found that in the absence of inducer, all of the cell lines can produce hCG- α —the trophoblastic tumour cells at roughly comparable rate, but the non-trophoblastic tumour cells at widely different rates (Fig. 1).

The basal production of complete hCG by the non-trophoblastic tumour cells is only marginal compared with its synthesis by the choriocarcinoma cells (Fig. 2). Sodium butyrate has strikingly different effects on the two classes of tumour cells: it induces synthesis of hCG- α and hCG in the non-trophoblastic tumour cells, but represses it in the trophoblastic tumour cells (Figs 1 and 2). Because of its differential effects on synthesis, we thought it possible that sodium butyrate might be more toxic for the choriocarcinoma cells. This is unlikely, however, for two reasons. Sodium butyrate inhibited the incorporation of uridine and leucine at roughly the same degree in JEG-3 and HeLa S₃ cells (Fig. 3). Although sodium butyrate slightly enhanced the incorporation of thymidine in JEG-3 cells but transiently inhibited the incorporation in HeLa S₃ cells, the long term effects seemed to be the same for both types of tumour cells (Fig. 3). The placental isoenzyme of alkaline phosphatase was induced by this compound in all cell lines examined (Fig. 4). Furthermore, since intracellular and extracellular hCG- α and

hCG are affected to the same extent by sodium butyrate (data not given), the observed reduction of these proteins in choriocarcinoma cells and induction in non-trophoblastic tumour cells are not due to its effects on hormone secretion.

Our data indicate that the regulation of hCG and hCG- α production differs in placental and non-placental tumours. Detailed analysis of the molecular mechanisms underlying the biosynthesis of this peptide hormone by these cell lines may yield insight into the essential nature of ectopic protein production.

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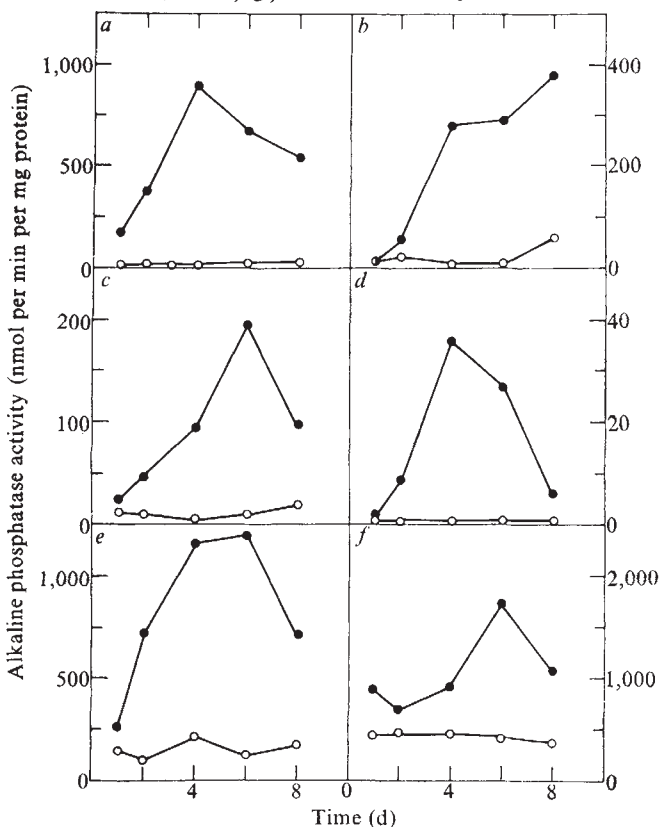
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Fig. 4 Effect of sodium butyrate on alkaline phosphate activity. *a–f* and conditions as in Fig. 1. Alkaline phosphatase activity in the sonicates was measured by release of *p*-nitrophenol from *p*-nitrophenyl phosphate at pH 10.7 and 37 °C (ref. 20). ○, Control; ●, 2 mM sodium butyrate.



Potent prolactin and growth hormone releasing activity of more analogues of Met-enkephalin

MORPHINE stimulates prolactin and growth hormone secretion^{1,2}, so the identification of brain peptides with opiate-like activity (methionine-enkephalin and leucine-enkephalin)³ raised the possibility that these peptides, beside their role as modulators of pain⁴, could also be involved in the control of neuro-endocrine functions. We did in fact find that Met-enkephalin administered intraventricularly led to a stimulation of prolactin and growth hormone release^{5–7}. β -Endorphin (β -LPH_{61–91}) was, however, found to be 500 to 2,000 times more potent than Met-enkephalin (β -LPH_{61–65}) in stimulating the release of the pituitary hormones. Since both peptides show similar affinity for the opiate receptor when binding studies are performed at 0 °C in conditions of minimal enzymatic degradation^{8,9}, the markedly different biological activities of the two peptides after intracerebral injection are likely to be due to a more rapid inactivation of Met-enkephalin. We show here that the two analogues resistant to enzymatic degradation, [D-Ala²]Met-enkephalin and [D-Ala²]Met-enkephalinamide can stimulate prolactin and growth hormone release after intraventricular injection at a level of potency which is 500 to 5,000 times higher than that of the natural Met-enkephalin molecule.

Since both plasma^{10,11} and brain tissue¹¹ contain high levels of enzymatic activity hydrolysing the Tyr¹-Gly² bond as the initial step of inactivation of Met-enkephalin, we first tested

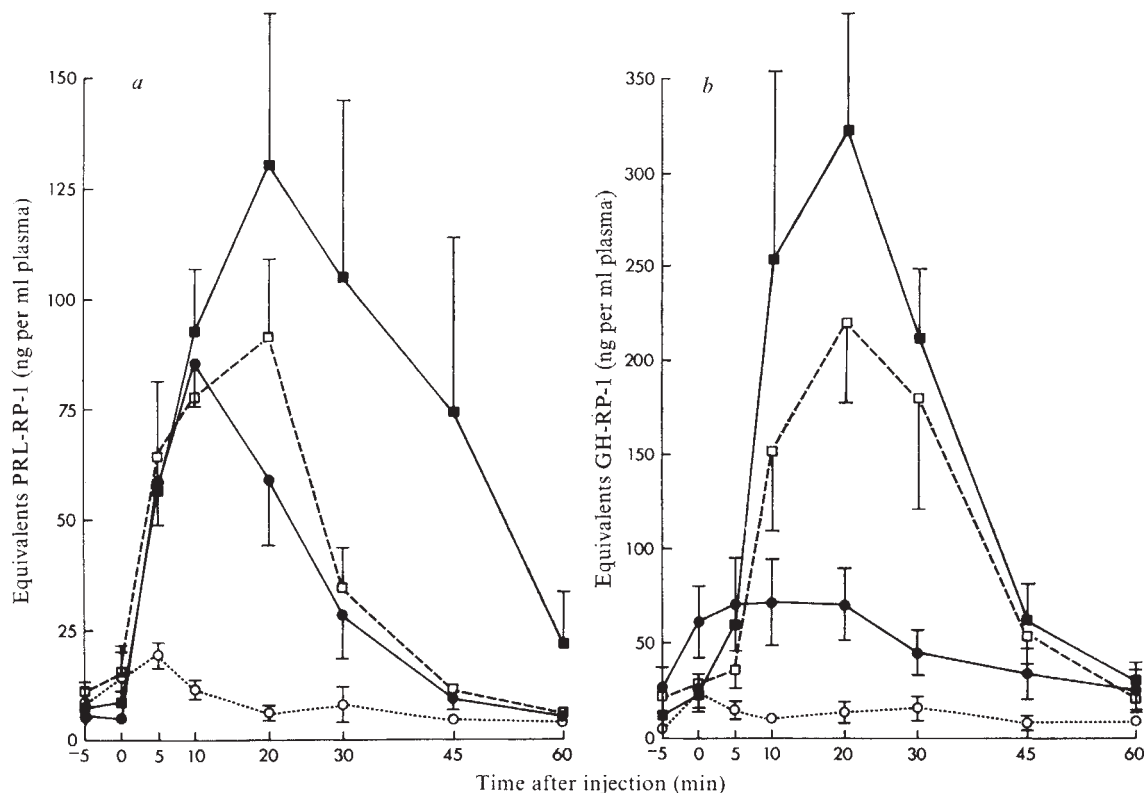


Fig. 1 Effect of increasing doses of Tyr-D-Ala-Gly-Phe-Met ([D-Ala²]Met-enkephalin) on plasma prolactin (a) and growth hormone (b) levels in the rat. To minimise stress-induced inhibition of growth hormone (GH) and stimulation of prolactin (PRL) release, adult male Sprague-Dawley rats (225–250 g) were handled twice a day for 7–10 d before insertion of a catheter into the right superior vena cava under Surital (50 mg per kg body weight, i.p.) anaesthesia. The animals were then implanted stereotactically with a stainless steel cannula (25 gauge) in the left lateral ventricle. Two days later, animals were injected through the intrajugular catheter with 0.5 ml of sheep anti-somatostatin serum 5 min before injection of the peptides to be tested which were injected in a volume of 40 µl of 0.9% NaCl over a 3-min period in freely-moving animals. Blood samples of 0.7 ml were then withdrawn at the indicated time intervals for measurement of plasma GH and PRL by double-antibody radioimmunoassays using materials provided by NIAMDD. Data are expressed as mean $\pm$ s.e.m. of triplicate determinations. Correct placement of the intracerebral cannulae was confirmed by injection of 10 µl of methylene blue at the time animals were killed. The peptides used were synthesised by solid-phase methods and purified as before¹². $\circ$, Control; $\bullet$, $\square$, $\blacksquare$; [D-Ala²]Met-enkephalin at 50, 150 and 300 µg respectively.

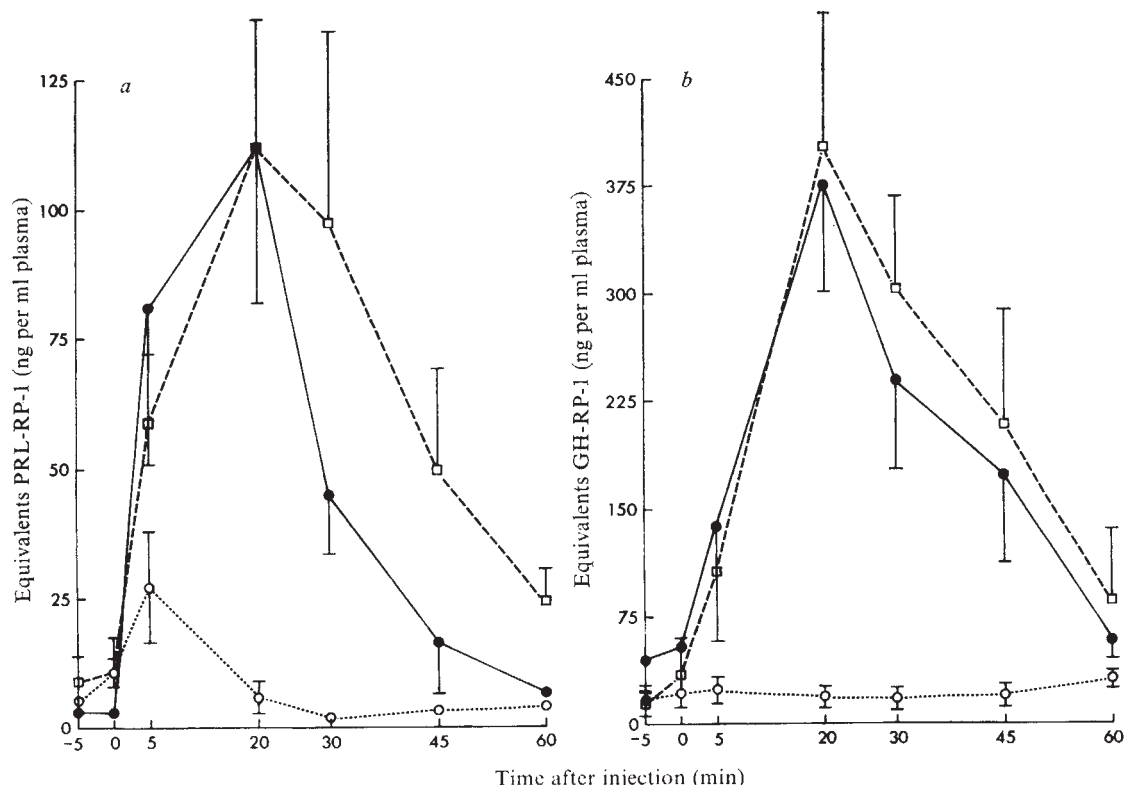


Fig. 2 Effect of intraventricular injection of 62 or 200 µg of [D-Ala²]Met-enkephalinamide on plasma prolactin (a) and growth hormone (b) levels in the rat. The experiment was performed in conscious freely-moving adult male animals bearing chronic intraventricular and intrajugular cannulae as for Fig. 1. $\circ$, Control; $\bullet$, 62.5 µg and $\square$, 200 µg [D-Ala²]Met-enkephalinamide.

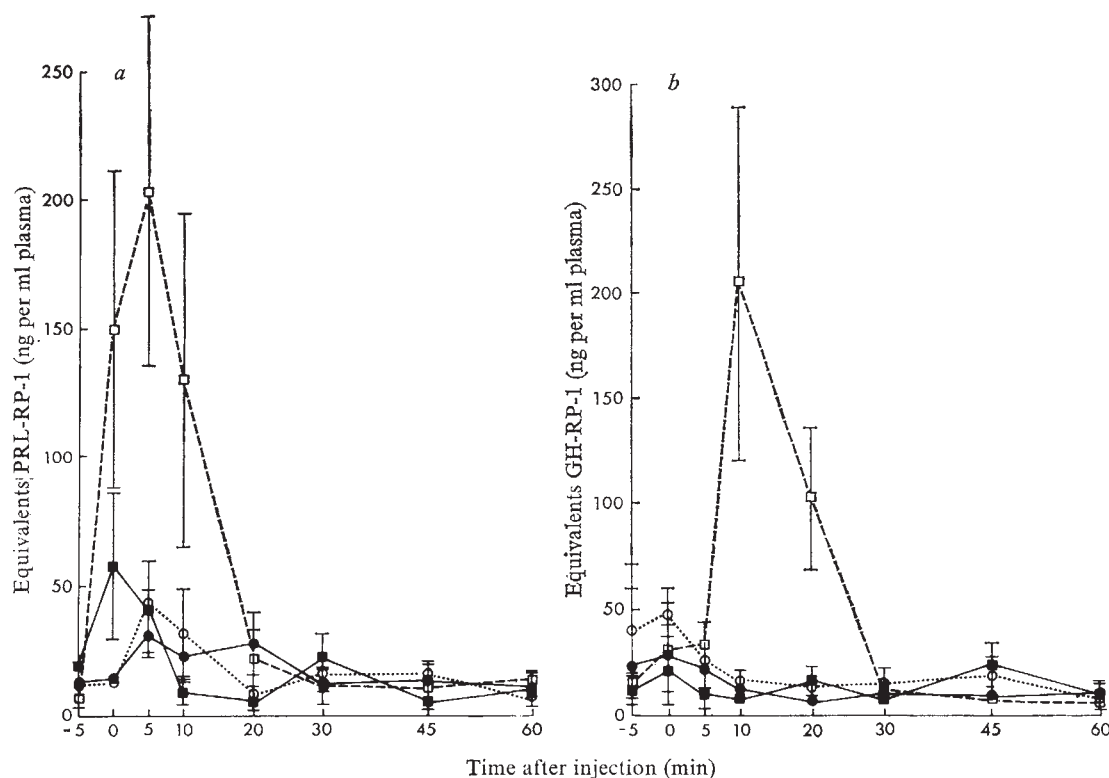


Fig. 3 Effect of intraventricular injection of 400 µg of *N*-acetyl [D-Ala²]Met-enkephalin (●), Met-enkephalin-ethylamide (□) or [D-Tyr¹]Met-enkephalin (■) on plasma prolactin (a) and growth hormone (b) levels in the rat. ○, Control. The experiment was performed in conscious freely-moving adult male animals bearing chronic intraventricular and intrajugular cannulae as described in Fig. 1.

the growth hormone and prolactin releasing activity of analogues of Met-enkephalin that seemed to be resistant to degradation, retained their activity for the opiate receptor^{8,9} and were found to be potent agonists in the vas deferens assay¹².

The minimal effective dose of Met-enkephalin on growth hormone and prolactin release after intraventricular injection in the rat is approximately 400 µg (refs 5–7), but it can be seen in Fig. 1 that [D-Ala²]Met-enkephalin has a maximal stimulatory effect on prolactin and growth hormone release at the doses of 50 and 150 µg, respectively. As observed previously with Met-enkephalin and β-endorphin^{5–7,20} the stimulatory effect of [D-Ala²]Met-enkephalin is exerted at a lower dose and earlier after its injection on prolactin than growth hormone release. In fact, while an important stimulatory effect of the Met-enkephalin analogue on plasma prolactin levels was seen 5 min after its injection, the effect on plasma growth hormone levels was detected 5 min later.

Substitution of D amino acids in position 6 of the decapeptide luteinising hormone-releasing hormone (LHRH) leads to peptides with much greater potency and duration of action^{13,14} while replacement of the tryptophan residue at position 8 in somatostatin by its D-isomer enhances the growth hormone-release inhibiting activity of the molecule approximately eightfold¹⁵. Our data show that substitution of the glycine residue of Met-enkephalin by D-alanine leads to a dramatic increase of the growth hormone and prolactin releasing potency of the peptide. Figure 1 indicates that [D-Ala²]Met-enkephalin given by intraventricular injection is 1,000 to 2,000 times more potent than Met-enkephalin itself.

Met-enkephalinamide has been reported to be slightly more effective than Met-enkephalin in an opiate receptor assay^{9,16}, so it is interesting to note that [D-Ala²]Met-enkephalinamide (Fig. 2) is about five times more potent than [D-Ala²]Met-enkephalin as stimulator of growth hormone and prolactin release. In fact, while maximal stimulation of prolactin and growth hormone release is obtained with [D-Ala²]Met-enkephalin at the 300-µg dose, a similar effect was obtained with

62.5 µg of [D-Ala²]Met-enkephalinamide, a peptide at least 5,000 times more potent than Met-enkephalin in this assay.

In agreement with our receptor binding studies^{9,12} and vas deferens bioassays¹², [D-Tyr¹]Met-enkephalin and *N*-acetyl Met-enkephalin did not show any growth hormone or prolactin releasing activity at the 400 µg dose, thus indicating the importance of the L-tyrosine residue for biological activity of the molecule. Substitution of the C-terminal glycine-amide residue in the LHRH molecule by ethylamide led to an analogue 10 to 15 times more potent than the natural hormone¹⁷. It is interesting to note that addition of the ethylamide group at the COOH-terminus of Met-enkephalin markedly increased its growth hormone and prolactin releasing activities (Fig. 3).

Our data show that two Met-enkephalin analogues studied have an activity comparable with that of β-endorphin itself as stimulators of growth hormone and prolactin release after intraventricular injection. These analogues should be useful for study of the physiological role of endogenous opiate peptides, not only as analgesics¹⁸ and behavioural modulators¹⁹ but also in the control of neuroendocrine functions. These data and previous work^{5–7,20} in fact suggest that endogenous opiate-like peptides are involved in the control of growth hormone and prolactin secretion. The model described here could also be useful for precise assessment of the biological activity of endogenous and synthetic opiate-like substances.

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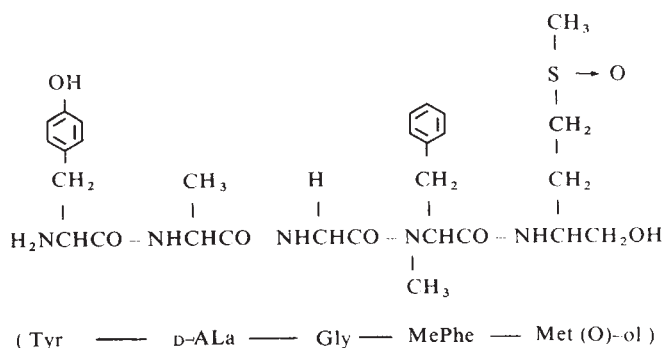


Fig. 1 Structural formula of 33-824

the rhesus monkey (shock titration test¹⁸⁻²⁰). The affinity for opiate receptors was assessed by the inhibition of ³H-naloxone binding to rat brain membranes using a method described previously⁴.

In the tail flick test (Table 1), met-enkephalin exhibited short-lasting analgesia following i.c.v. and i.v. administration⁴. A gradual increase of analgesic potency and duration after i.c.v. administration resulted from the alteration of the carboxyl group to the carbinol analogue (Met⁵-ol), the substitution of the glycine residue in position 2 by D-alanine (D-Ala², Met⁶), and the combination of these two modifications (D-Ala², Met⁵-ol). The latter derivative showed subcutaneous as well as prolonged i.c.v. and i.v. analgesic activity. In contrast, the corresponding (D-Ala², Met⁶) enkephalin amide²¹ was only active following i.c.v. administration. Oxidation to the sulphoxide (D-Ala²Met(O)⁵-ol) and *N*-methylation of the phenylalanine residue gave compound FK 33-824 (referred to throughout as 33-824), (D-Ala², MePhe⁴, Met(O)⁵-ol)-enkephalin (Fig. 1), which produced definite analgesic activity even after oral administration. 33-824 was, on a molar basis, not only about 30,000 and 1,000 times more potent than met-enkephalin and morphine respectively after i.c.v. administration, but also 23 times as active as β -endorphin.

In binding studies using ^3H -naloxone²² and rat brain membranes (Table 1) met-enkephalin and its Met⁵-ol derivative had a greater affinity for the opiate receptor in the presence of the enzyme inhibitor²³ bacitracin than in its absence, indicating that even in this *in vitro* system, both compounds are subjected to inactivation. For all the other compounds including morphine, the dose-response curves for opiate-receptor binding were not affected or were shifted only slightly to the right, suggesting that these compounds are sufficiently stable in the *in vitro* conditions used. In agreement with the *in vivo* results,

A synthetic enkephalin analogue with prolonged parenteral and oral analgesic activity

METHIONINE and leucine enkephalin, the morphinomimetic pentapeptides from mammalian brain¹⁻³ produce a weak and short-lived analgesia following intracerebroventricular (i.c.v.) or intravenous (i.v.) administration to mice⁴ and rats⁵. β -endorphin^{6,7}, another morphinomimetic fragment of β -lipotropin but with 31 amino acid residues, shares its amino-terminal sequence with met-enkephalin. In addition to showing marked and long-lived analgesic activity following i.c.v. and i.v. administration⁸⁻¹⁰, the pronounced effects of β -endorphin have led to the suggestion that it may be implicated in the aetiology of mental illness¹¹⁻¹³. We report here a number of the pharmacological properties of five synthetic pentapeptides structurally related to met-enkephalin, two of which exhibit significant analgesic activity after oral administration and which are more potent analgesics administered parenterally than β -endorphin.

The enkephalin analogues were prepared by fragment condensation methods. Analgesia was estimated in the mouse (tail flick¹⁴ and hot plate^{15,16} procedures), the rat (paw pressure test based on the technique described by Randall and Selitto¹⁷) and

Table 1 Analgesic activity in the mouse and inhibition of ^3H -naloxone binding to rat brain membranes

Compound	Tail-flick test ED ₅₀					Relative molar dose i.c.v.	IC ₅₀ (nM) of specific ³ H-naloxone binding	
	i.c.v. 15 min (µg per mouse)	i.v. 15 s (mg kg ⁻¹)	i.v. 30 min (mg kg ⁻¹)	s.c. 30 min (mg kg ⁻¹)	p.o. 120 min (mg kg ⁻¹)		Without bacitracin	Bacitracin (0.1 mg ml ⁻¹)
Met-enkephalin	68*	153				1	412	138
Met ⁵ -ol	43	52				0.6	50	27
D-Ala ² , Met ⁵	0.7	51				0.01	61	132
D-Ala ² , Met ⁵ -ol	0.04	12	13	59	> 640	0.0006	20	31
D-Ala ² , Met(O) ⁵ -ol	0.01	12	3.5	3.9	319	0.0001	60	95
D-Ala ² , MePhe ¹ , Met-(O) ⁵ -ol (33-824)	0.002	0.7	0.4	1.4	102	0.00003	12	14
β-Endorphin	0.3					0.0007	35	35
Morphine-HCl	1.0		1.8†	3.0	20.5‡	0.03	73	83

*At 2 min: †at 15 min: ‡at 30 min.

The ED₅₀ value³³ for the analgesic activity in the tail-flick test is defined as the dose that prolonged the reaction time (in s) by more than 75% in half the animals. Ten animals were used per dose. All doses refer to the free base. The IC₅₀ is the concentration of substance required to inhibit by 50% the specific binding of ³H-naloxone (1 nM) to pre-incubated (30 min at 37 °C) membranes of rat brain minus cerebellum in 0.05 M Tris buffer (pH 7.4 at 25 °C). Five concentrations of each compound were incubated in triplicate at 25 °C for 40 min in the presence of 100 mM NaCl (refs 4, 25). Filtrations were performed at 25 °C. Specific binding is the difference between ³H-naloxone binding in the absence and presence of 1 μM levallorphan. Values are the mean of at least three determinations with s.e.m. of less than 15%.

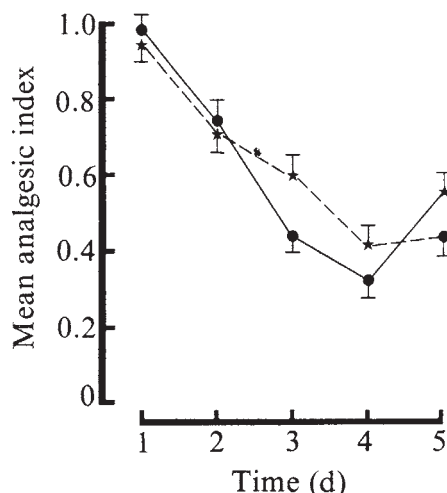


Fig. 2 Mean analgesic index ($\pm$ s.e.) recorded in the paw pressure test in the rat²⁸ following s.c. administration of morphine (7.5 mg kg^{-1} , ●) and 33-824 (3.2 mg kg^{-1} , ★) twice daily for 4 d. On day 5, 33-824 (3.2 mg kg^{-1} s.c.) was given to the morphine-pretreated rats and morphine (7.5 mg kg^{-1} s.c.) to the 33-824-pretreated animals. Twenty rats were in each group. The reaction thresholds were recorded immediately before and 30 min after each injection (0815 h and 1545 h).

33-824 displayed the highest affinity for the opiate receptor. The data in Table 1 indicate that in the absence of bacitracin an approximately 30-fold increase in affinity was observed between the least (met-enkephalin) and the most (33-824) potent compound in this series. This increase is much smaller than the dramatic, graded increase observed *in vivo*. The 'sodium response ratio' for 33-824, calculated from the ID_{50} values obtained in the presence and the absence (data not shown) of NaCl (ref. 24), was 8.2, thereby placing it in the same range as the partial morphine agonist pentazocine which had a ratio of 7.1 (ref. 4). Since there is *in vivo* no evidence to suggest that 33-824 possesses morphine antagonistic properties, the value of the 'sodium response ratio' as a predictor²⁴ of the degree of morphine-like activity of opioid peptides remains uncertain²⁵.

In a more detailed comparison of the analgesic properties of 33-824 and morphine (Table 2), 33-824 was about twice as potent subcutaneously in the mouse and rat. Thirty minutes after oral administration, however, 33-824 was 5 (tail flick) and 3.5 times (hot plate) less active than morphine. The similarity of the ED_{50} s obtained with 33-824 in the inflamed and non-inflamed paws in the paw pressure test confirm the central nature of its analgesic effect. Aspirin, with a predominantly peripheral analgesic effect²⁶, only affects the threshold in the inflamed paw. In the rhesus monkey, 33-824 was parenterally about 2–3 times less potent than morphine, depending on the route of administration. Following rectal administration, however, 33-824 was active whereas no analgesic effect was observed with

morphine. The degree and duration of analgesia with 33-824 was dose dependent in all species investigated. In the mouse (tail flick) the ED_{50} s after i.v. administration at 0.5 and 2 h were 0.4 and 0.8 mg kg^{-1} respectively. Orally, in the same test, the ED_{50} increased between 2 and 3 h after administration from 102 to 114 mg kg^{-1} . In the rhesus monkey the duration of action following i.v. administration of the minimal effective dose was at least 5 h.

The parallel displacement to the right of the analgesic dose-response curves of 33-824 and morphine in the tail-flick test in a manner similar to that obtained with methionine enkephalin⁴ following pretreatment with naloxone (0.56 mg kg^{-1} i.v.), provide evidence for the morphine-like qualities of 33-824. This was confirmed by the ability of 33-824 to produce mydriasis and a Straub tail response in the mouse, that were abolished by naloxone administration. Repeated administration of 33-824 to rats twice daily for four consecutive days²⁷ resulted in the development of tolerance to its analgesic effect in the paw pressure test and cross-tolerance with the analgesic effect of morphine given on day 5 (Fig. 2). In rats similarly pretreated with morphine, 33-824 exhibited a markedly reduced analgesic effect (Fig. 2).

In the single dose suppression test²⁸ in morphine-dependent rhesus monkeys (3.2 or 5.6 mg kg^{-1} i.v. 6 times daily for a minimum of 4 weeks), 33-824 (1.8 – 5.6 mg kg^{-1} i.v.) abolished the morphine withdrawal syndrome. Substitution of morphine injections in morphine-dependent, non-withdrawn rhesus monkeys by 33-824 (1.8 mg kg^{-1} i.v. at 4 h intervals for 24 h) prevented the appearance of the morphine withdrawal syndrome. In a self-administration study in three male, drug-naïve rhesus monkeys²⁹, 33-824 (0.01 and 0.032 mg kg^{-1} i.v. per injection), was not self-administered in spite of continual access to the drug for a total of 4 weeks. When the concentration was increased to 0.1 mg kg^{-1} i.v. per injection, all three animals started to exhibit self-administration behaviour which increased gradually over a period of 5 weeks, until the mean daily amount self administered was about 13 mg kg^{-1} i.v. (Table 3). Naloxone (i.v.) produced a marked withdrawal syndrome resembling that observed following opiate withdrawal and saline substitution for 33-824 injections resulted in all three monkeys in the occurrence of a marked opiate-like withdrawal syndrome lasting 2–3 d and accompanied during the first 48 h by an increase in lever-pressing activity (Table 3).

33-824 (10 mg kg^{-1} i.p.) was devoid of morphine-antagonistic properties in the mouse as, in contrast to naloxone (0.56 mg kg^{-1} i.p.), it did not produce jumping behaviour in morphine-dependent³⁰ mice. Thus, 33-824 may be regarded at this stage as being a pure morphinomimetic drug without any antagonistic properties.

33-824 ($10 \mu\text{g}$ per rabbit) administered into the 4th ventricle produced a marked catatonia characterised by muscular rigidity and immobility lasting at least 2 h and which had the same outward appearance as that exhibited by β -endorphin in rats^{11,12}. Morphine (1 mg per rabbit in 0.1 ml) injected directly into the 4th ventricle caused marked respiratory depression

Table 2 Analgesic activity of 33-824 and morphine

Compound	Tail flick (mouse)		ED_{50} (mg kg^{-1}) Hot plate* (mouse)		Paw pressure† (rat)		Minimal effective dose (mg kg^{-1}) Shock titration (rhesus monkey)		
	s.c.	oral	s.c.	oral	s.c.	s.c.	i.v.	s.c.	rectal
33-824	1.4	102	1.1	20.5	0.7	0.9	1.8	1.8	56
Morphine-HCl	3.0	20.5	2.1	5.8	1.3	2.0	1.0	0.56	> 100

*At 60 min; † at 30 min.

ED_{50} values³³ for the analgesic activity in the tail-flick, hot plate, and paw pressure tests are defined as the doses that prolonged either the reaction time (in s) or the reaction pressure (mmHg) by more than 75% in half of the animals. Ten animals were used per dose. The minimal effective dose in the shock titration test is the dose that increased the mean tolerated threshold by more than 45% in three of six post-treatment sessions. All doses refer to the free base.

Table 3 Self-administration of 33-824 in the rhesus monkey

Treatment	Time (d)	Mean daily values (24 h)		Mean daily dose (mg kg ⁻¹ i.v.)
		Lever presses	Injections	
NaCl (0.2 ml kg ⁻¹ per injection)	7	91	30	—
33-824 (0.1 mg kg ⁻¹ per injection + Pellet reward*)	4	183 (163†)	32 (21†)	3.2
33-824 (0.1 mg kg ⁻¹ per injection)	3	24	17	1.7
33-824 (0.1 mg kg ⁻¹ per injection)	7	40	30	3.0
33-824 (0.1 mg kg ⁻¹ per injection)	7	105	61	6.1
33-824 (0.1 mg kg ⁻¹ per injection)	7	155	99	9.9
33-824 (0.1 mg kg ⁻¹ per injection†)	7	204	136	13.6
NaCl (0.2 ml kg ⁻¹ per injection)	1	719	391	—
NaCl (0.2 ml kg ⁻¹ per injection)	1	249	110	—
NaCl (0.2 ml kg ⁻¹ per injection)	1	192	120	—

* 15-min period daily in which the animals had simultaneous access to drug and 75 mg food pellets.

† Mean number of lever presses made and injections of drug received during 15-min food pellet access period.

‡ Naloxone (0.1 mg kg⁻¹ i.v.) administered once on day 1 of this week.

and death but no catatonia, thereby confirming the results obtained in a similar study in the rat.¹¹ 33-824 (32 mg kg⁻¹ s.c.) also caused catatonia in the rat. As in the case of β -endorphin^{9,11}, the catatonic effects of 33-824 were abolished by naloxone. β -Endorphin also resembles 33-824 in that it produces tolerance to its own analgesic effect³¹, exhibits cross tolerance with morphine and causes physical dependence³².

The major finding of the present studies is that by appropriate modification of enkephalin it is possible to construct a synthetic pentapeptide possessing many of the biological properties exhibited by the untriacontapeptide β -endorphin. The outstanding characteristic of 33-824 is that its long-lasting analgesic effect after systemic administration is superior to that of β -endorphin. Furthermore, 33-824 is one of the rare examples of a peptide showing pronounced biological activity after enteral administration. The ready availability and the stability of 33-824 should make it valuable for further studies of the mode of action of opioid peptides.

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Opiate analgesics inhibit substance P release from rat trigeminal nucleus

THE undecapeptide substance P (SP) may act as a transmitter released from the terminals of primary sensory nerves in the spinal cord¹⁻². SP has been shown to be highly concentrated in the terminals of small diameter fibres in the substantia gelatinosa of the dorsal horn in mammalian spinal cord, and these terminals disappear after dorsal root section³⁻⁶. Sensory nuclei of some of the cranial nerves, such as the spinal trigeminal nucleus, also contain a high density of SP nerve terminals⁶⁻⁷. SP is released by a calcium-dependent mechanism from the isolated rat spinal cord during electrical stimulation of the dorsal roots⁸. The peptide is also a powerful excitant of neurones in the spinal cord², and seems to excite selectively those cells in dorsal horn that respond to noxious stimuli⁹⁻¹⁰. SP may, therefore, represent the excitatory transmitter, or one of the transmitters, released from primary fibres involved in the transmission of pain. We have examined the stimulus-evoked release of SP from rat spinal trigeminal nucleus slices *in vitro* to explore the factors which may control its release from primary sensory terminals. We report here that opiate analgesics are able to suppress the stimulus-evoked release of SP. These findings may represent a mechanism for the direct spinal analgesic actions of opiates.

In the mammalian spinal cord and cranial nerve nuclei, there is a striking similarity between the anatomical distributions of opiate receptor binding sites and the endogenous opioid peptide, enkephalin¹¹⁻¹². The results of SP assays on microdissected areas of rat brain stem and spinal cord show that SP is similarly distributed (Fig. 1).

For release studies we used the techniques previously developed for studying the calcium-dependent release of SP from slices of rat hypothalamus or substantia nigra *in vitro*^{13,14}. Spinal trigeminal nerve nuclei were dissected from fresh chilled 0.8 mm thick coronal sections of rat brain stem, using a stereomicroscope. The pooled tissue samples from several rats (approximately 10 mg wet weight from one rat brain) were sliced at 0.2-mm intervals in two directions with a McIlwain tissue chopper, and approximately 20 mg of tissue was transferred to each chamber of a four-channel superfusion apparatus¹³. After washing (0.375 ml min⁻¹) for 5 min with Krebs bicarbonate solution at 37 °C containing 0.5% bovine serum albumin and the peptidase inhibitor bacitracin (20 μ M), superfusate samples were collected at 1-min intervals and SP-like immunoreactivity measured as described previously¹³. The spontaneous efflux of SP was equivalent to 0.2-0.4% of the total tissue content/min. As in previous experiments with slices of hypothalamus or substantia nigra^{13,14}, brief exposure to a medium containing 47 mM potassium chloride (K⁺)

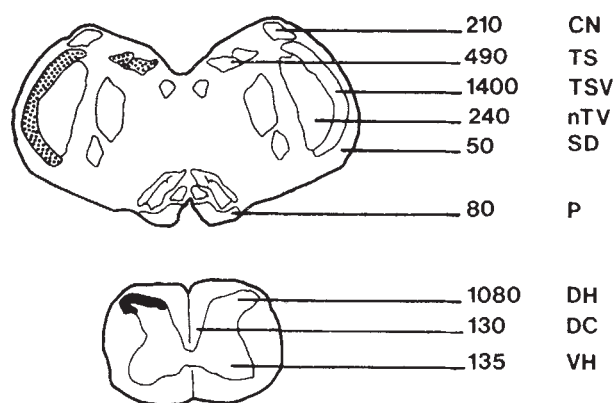


Fig. 1 Comparative distribution of opiate receptor binding and SP in rat brain stem and spinal cord. Diagrammatic representation of coronal sections of (a) rat medulla, 2 mm caudal to the obex; and (b) rat cervical spinal cord. Opiate receptor binding data are adapted from Atweh and Kuhar¹¹. The left-hand side of each section shows areas with dense (solid black) or moderate (stippled) opiate receptor binding. The right-hand side of each section gives the SP concentrations expressed as pmol per g wet weight in areas obtained by microdissection. Each value is the mean $\pm$ s.e.m. of at least four animals (measured by radioimmunoassay as described previously⁴). CN, Nucleus cuneatus; TS, nucleus tractus solitarius; TSV, tractus spinalis nervi trigemini (substantia gelatinosa); nTV, nucleus tractus spinalis nervi trigemini; SD, tractus spino-cerebellaris dorsalis; P, pyramis; DH, dorsal horn; DC, dorsal column; VH, ventral horn.

elicited a marked increase in the release of immunoreactive SP, with efflux returning to basal levels soon after removal of K^+ (Fig. 2a). Addition of morphine to the superfusing medium for 7 min before K^+ application produced a concentration-dependent inhibition of the evoked release of SP, without affecting the spontaneous efflux (Fig. 2b). At a morphine concentration of $10 \mu M$, the K^+ -evoked release of SP was almost

completely suppressed. Continuous superfusion with naloxone ($1 \mu M$) commencing 2 min before addition of $10 \mu M$ morphine caused an almost complete reversal of the effect of morphine on SP release (Fig. 2c). Superfusion with naloxone alone ($1 \mu M$) failed to affect the spontaneous efflux of SP or the K^+ -evoked release.

The opiate effect was stereospecific in that levorphanol ($5 \mu M$) produced an almost complete inhibition of the K^+ -evoked release of SP, whereas the inactive enantiomer dextrorphan did not significantly inhibit SP release at a tenfold higher concentration ($50 \mu M$) (Fig. 2d). Another opiate analgesic, normorphine ($5 \mu M$), similarly suppressed the K^+ -evoked release of SP, and this effect could be partially reversed by addition of the potent opiate antagonist diprenorphine ($1 \mu M$) 2 min before addition of normorphine (Fig. 2e).

A number of naturally occurring peptides with morphine-like properties, the endorphins, have been described¹⁵⁻¹⁸, which vary considerably in metabolic stability and analgesic potency. In the present studies we tested the Met-enkephalin analogue [D-Ala]²-Met-enkephalin-amide (Peninsula Laboratories), which is reported to be metabolically stable and to possess high analgesic potency¹⁷. A low concentration of this peptide ($3 \mu M$) almost completely suppressed the K^+ -evoked release of SP, and this inhibitory effect was reversed by naloxone ($1 \mu M$) (Fig. 2f). In other experiments (results not shown), another opioid peptide with similar metabolic stability and high analgesic potency¹⁸, β -endorphin (Peninsula Laboratories), was found to produce $43.3 \pm 8.1\%$ (mean $\pm$ s.e.m., $n = 4$) inhibition of K^+ -evoked SP release at an even lower concentration ($0.5 \mu M$).

We also examined the effects of morphine and β -endorphin on the release of SP from slices of rat substantia nigra, a region of rat brain that contains a concentration of SP comparable with that found in the substantia gelatinosa of the spinal nucleus of the trigeminal nerve⁴. No significant inhibition of the spontaneous or K^+ -evoked release of SP from nigral tissue was observed with morphine or β -endorphin. In control experiments, exposure to K^+ evoked a total increase in SP release equivalent

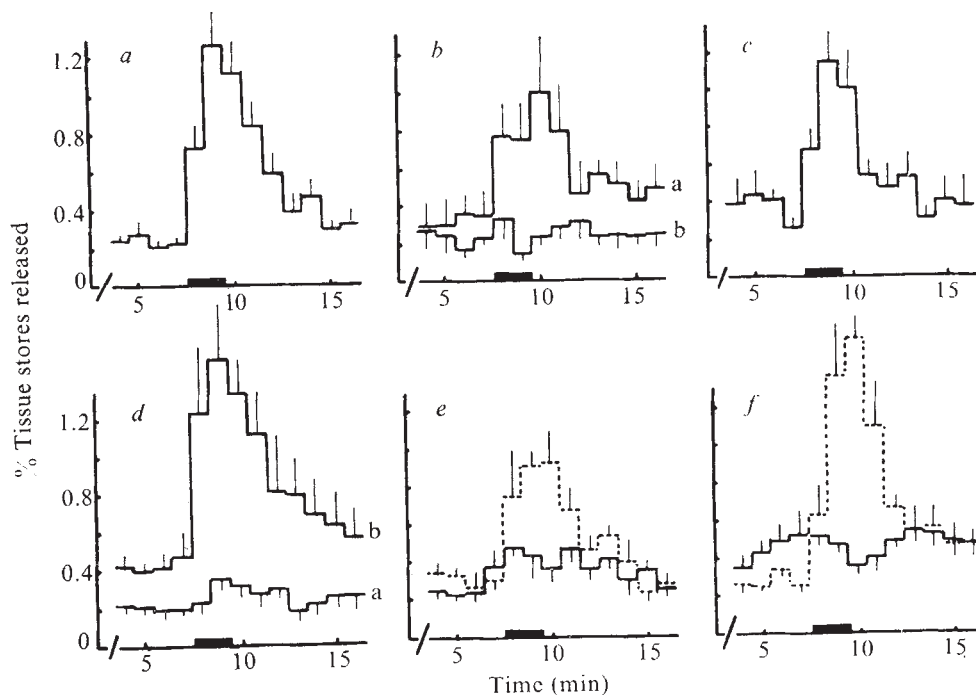


Fig. 2 Effect of opiates on the release of SP from superfused slices of spinal trigeminal nucleus from rat brain. Efflux of SP is expressed as the percentage of tissue content released per min. The spontaneous efflux of SP was approximately 30 fmol per fraction and the lower limit of sensitivity of the radioimmunoassay procedure was approximately 8 fmol. In a-f, each point is the mean $\pm$ s.e.m. of at least four experiments. Tissue slices were exposed to KCl-enriched Krebs solution (47 mM) for a 2-min period (indicated by horizontal black bar). a, Control (K^+ -evoked release of SP from superfused slices of spinal trigeminal nucleus); b, inhibition of K^+ -evoked release of SP by morphine (upper: $5 \times 10^{-6} M$; lower: $10^{-6} M$); c, naloxone ($10^{-6} M$) reversal of morphine-induced ($10^{-6} M$) inhibition of K^+ -evoked SP release; d, effect of levorphanol ($5 \times 10^{-6} M$: a) and dextrorphan ($5 \times 10^{-5} M$: b) on K^+ -evoked SP release; e, inhibition of K^+ -evoked SP release by normorphine (continuous line; $5 \times 10^{-6} M$) and reversal by diprenorphine (dotted line; $10^{-6} M$); f, inhibition of K^+ -evoked SP release by [D-Ala]²-Met-enkephalin amide ($3 \times 10^{-6} M$) (continuous line) and reversal by naloxone ($10^{-6} M$) (broken line).

to $2.57 \pm 0.26\%$ (mean $\pm$ s.e.m.) of total tissue stores ($n = 6$). In slices exposed to $10 \mu\text{M}$ morphine, K^+ -evoked release was $2.67 \pm 0.29\%$ ($n = 4$) and in two experiments with $0.5 \mu\text{M}$ β -endorphin the K^+ -evoked release was $2.54 - 2.79\%$. Thus, opiates do not inhibit SP release from all SP-containing regions of CNS.

We believe that the present results can most easily be explained by postulating that some opiate receptors are located on primary afferent nerve terminals containing SP in the substantia gelatinosa. This would be consistent with previous work which has shown that opiates can act presynaptically to inhibit neurotransmitter release from a variety of other neurones (see, for example, ref. 19), and with the known distribution of enkephalins and opiate receptors in brain stem and spinal cord. Thus, after dorsal rhizotomy there is no change in the enkephalin content of the substantia gelatinosa area²⁰ but there is a significant decrease in the number of opiate receptor binding sites²¹, suggesting that some of these receptors may be located on the presynaptic terminals of primary afferent neurones. Furthermore, recent immunohistochemical findings suggest that enkephalin-containing local interneurons are abundant in the substantia gelatinosa area of spinal cord and in the spinal trigeminal nerve nucleus^{12,20}, which represents a rostral extension of the dorsal horn, involved in the transmission of nociceptive and other information from the facial area.

The cellular mechanism by which opiates and a variety of other agents inhibit the release of transmitters from presynaptic nerve terminals remains obscure²². In most cases, as in the present results, control is exerted on the stimulus-evoked release of transmitter rather than on basal efflux, suggesting some interaction with the stimulus-secretion coupling mechanism, for example blockage of calcium influx²².

A possible neuronal basis for the opiate-mediated presynaptic inhibition of primary afferent terminals and the role of enkephalins at the level of the brain stem and spinal cord is shown schematically in Fig. 3. A mechanism of this nature could account for the selective blockade by opiates of the transmission of nociceptive information in the dorsal horn²³⁻²⁵, and their direct spinal analgesic actions²⁶. This model could also represent the morphological substrate of the gating process proposed

by Melzack and Wall²⁷ for the modulation of the transmission of nociceptive information within the substantia gelatinosa.

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Alterations in dopamine receptor sensitivity by chronic ethanol treatment

THE hypothermic effect of an acute dose of ethanol in animals is well established^{1, 2}, and the development of functional tolerance to ethanol can be monitored by measuring decreased responsiveness to the hypothermia produced by ethanol³. Although the central neuronal systems mediating the ethanol-induced hypothermia are at present unknown, it has been found that hypothermia caused by apomorphine and pibedil in mice and rats^{4, 5} is mediated by the action of these drugs on the central dopamine receptors. Since single doses of ethanol can increase the synthesis^{6, 7} and/or release⁸ of dopamine in brain, the possibility exists that ethanol-induced hypothermia in rodents may be in part related to activation of dopaminergic systems. If this is the case, an altered sensitivity of dopamine receptors during chronic treatment with ethanol may be responsible for the resultant decreased sensitivity to the effects of ethanol which accompanies the development of tolerance. This paper presents evidence consistent with that possibility.

Male C57B1/6 mice were fed a liquid diet containing 7% v/v ethanol or an equicaloric amount of sucrose (controls) for 7 d (ref. 3). At the conclusion of the drinking period, all mice were offered only the sucrose-containing liquid diet (withdrawal). Using this regimen, we have previously shown that tolerance to the hypothermic and behavioural effects of ethanol is clearly evident after the 7 d of drinking and continues for up to 5 d after withdrawal³. In contrast, withdrawal symptoms are no longer evident at 24 h after withdrawal³. In the present study, tolerance to the hypothermic and hypnotic effects of ethanol was determined as previously described³ at 24 h and at 3 and 7 d after withdrawal. At these same times, the hypothermic response to intraperitoneal (i.p.) administration of a dopamine agonist, pibedil (1-(2-pyrimidyl)-4-piperonyl-piperazine)⁹ was assessed. To further monitor dopaminergic

Fig. 3 Schematic representation of a possible mechanism for opiate-induced suppression of SP release. SP is shown localised within the terminal of a small diameter afferent fibre which forms an excitatory axodendritic synapse with the process of a spinal cord neurone originating in lamina IV or V and projecting rostrally. A local enkephalin-containing inhibitory interneurone (ENK), confined to laminae II and III forms a presynaptic contact on the terminal of the primary afferent. Opiate receptor sites are depicted presynaptically on the primary afferent terminal. Numbers on the right refer to the laminae of Rexed.

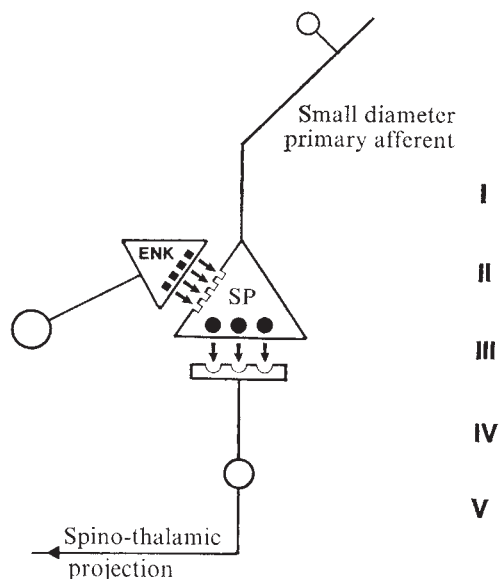


Table 1 Change in body temperature of C57B1/6 mice after drug treatment*

Drug treatment (per kg bodyweight)	Ethanol-tolerant	Control	P†
Ethanol, 3 g	1.5±0.5 (6)	3.5±0.4 (6)	<0.01
Piribedil 20 mg	1.2±0.4 (5)	2.2±0.3 (4)	<0.1
40 mg	2.0±0.1 (6)	2.9±0.1 (6)	<0.001
60 mg	2.7±0.1 (6)	3.4±0.1 (6)	<0.001
Clonidine 0.5 mg	3.9±0.4 (4)	3.4±0.5 (4)	NS

Body temperature (°C) was monitored at 15-min intervals for 30 min before i.p. injection of the drug, and at 15-min intervals for 2 h after the drug treatment. The values recorded represent the decrease in body temperature ± s.e.m. from the pre-injection baseline at 30 min after drug treatment, the time of maximum change. Rectal temperature was measured by insertion of a thermometer probe (Yellow Springs Instrument Company) 2.5 cm into the rectum. Values in parentheses represent number of animals tested.

*Ethanol-tolerant animals had been withdrawn from ethanol for 24 h.

†Values were compared by using Student's *t* test.

function, the dopamine-sensitive adenylate cyclase in brain was assayed for several days after ethanol withdrawal. Dopamine-sensitive adenylate cyclase activity was also measured in certain animals 8 h after a single i.p. injection of ethanol at 3 g per kg bodyweight. The brain section used for measurement of adenylate cyclase activity comprised the corpus striatum, diencephalon, and limbic areas⁶. This section was homogenised and assayed as described by Keabian *et al.*¹⁰ except that α -³²P-ATP was used as substrate and ³²P-cyclic AMP was isolated according to Saloman *et al.*¹¹. The hypothermic effect of clonidine, an α -adrenergic agonist, was also monitored in both control and ethanol-tolerant animals.

Table 1 shows that animals which have been withdrawn from the ethanol-containing diet for 24 h were tolerant to the hypothermic effect of an acute dose of ethanol and were also less sensitive than controls to the hypothermic effect of piribedil. In contrast, ethanol-tolerant and control animals were equally sensitive to the hypothermic effects of the α -adrenergic stimulant clonidine. The change in the dose-response pattern of the hypothermic effects of piribedil in the ethanol-tolerant animals suggested a decrease in central dopaminergic receptor responsiveness to this agonist.

The dopamine-sensitive adenylate cyclase has been reported in most instances¹² to have many of the characteristics of the dopamine receptor *in vivo*^{10, 13}. At 8 h after injection of a single dose of 3 g per kg ethanol, we found that basal adenylate cyclase activity and the stimulation of the enzyme by dopamine was similar in brains of ethanol-treated and control animals (that is at 10^{-5} M dopamine, the percentage stimulation over basal values was 66.8 ± 9.1 (s.e.m.) in ethanol-treated and 66.4 ± 3.9 in control animals). On the other hand, Fig. 1 illustrates the response of the dopamine-sensitive adenylate cyclase of mouse brain to various doses of dopamine in control animals and animals fed the ethanol-containing diet for 7 d and withdrawn for 24 h. The adenylate cyclase from the ethanol-tolerant animals showed a decreased response at all dopamine concentrations tested, and the response did not seem to reach the same maximum value as seen in control animals. Basal values were the same in control and ethanol-tolerant animals (see legend to Fig. 1).

The data in Table 2 demonstrate that the decreased sensitivity to the hypothermic effect of both ethanol and piribedil was still present at three days after ethanol withdrawal, but was no longer evident by 7 d after withdrawal. Similarly, dopamine-sensitive adenylate cyclase from ethanol-tolerant animals was significantly less sensitive to dopamine stimulation at three, but not at 7 d after withdrawal (see Table 2).

Seerber and Kuschinsky¹⁴ have demonstrated that the dopamine-sensitive adenylate cyclase in brains of rats withdrawn from chronic barbiturate treatment was less responsive to dopamine stimulation than enzyme from control animals. These authors,¹⁴ however, found little difference in response

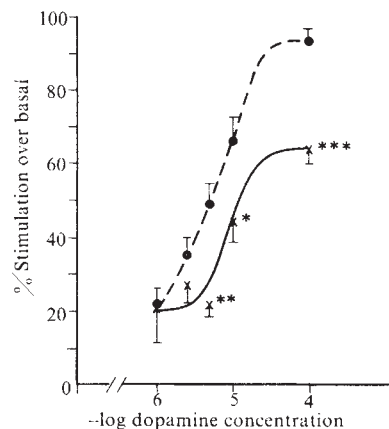


Fig. 1 Stimulation of dopamine-sensitive adenylate cyclase in brain of control (●) and ethanol-tolerant (×) animals. Animals were killed 24 h after withdrawal from the ethanol-containing diet, after 7 d of consumption. Brains were dissected and dopamine-sensitive adenylate cyclase activity was measured as described in the text. Basal values for control and ethanol-tolerant animals were not significantly different (respectively, 31.5 ± 3.5 and 29.9 ± 3.1 pmol cyclic AMP produced per 3 min). Dopamine concentration is molar, and results are expressed as per cent stimulation over basal values ± s.e.m. Values which are significantly different in ethanol-tolerant and control animals are indicated as follows: **P* < 0.05; ***P* < 0.01; ****P* < 0.001 (*t* test).

to dopamine between ethanol-treated and control animals, but their animals were not shown to have developed any tolerance to ethanol, and the development of central nervous system tolerance may be critical for the observed alterations in adenylate cyclase activity.

Our finding that the maximal response of the adenylate cyclase to dopamine is decreased in ethanol-tolerant animals, while the concentration of dopamine producing a half-maximal response seems similar in both control and ethanol-treated animals, is in line with previous suggestions that changes in receptor sensitivity are related to alterations in receptor density rather than affinity for agonist^{15, 16}.

We have recently demonstrated that partial destruction of brain noradrenergic systems by 6-hydroxydopamine (6-OHDA) before ethanol consumption blocked the development of tolerance to ethanol¹⁷, but the same treatment administered

Table 2 Time course for the disappearance of ethanol tolerance and of alterations in dopamine receptor sensitivity

Drug	Time after withdrawal (d)	Change in body temperature			P*
		Ethanol-tolerant	Control		
EtOH 3 g per kg	3	1.8±0.2 (6)	3.5±0.2 (6)		<0.05
	7	3.9±0.2 (6)	3.3±0.3 (6)		NS
Piribedil	3	0.5±0.2 (3)	1.8±0.6 (3)		<0.1
	7	1.8±0.2 (8)	2.0±0.2 (8)		NS
	Time after withdrawal (d)	Adenylate cyclase activity, % stimulation			
		Ethanol-tolerant	Control	Δ	P
Dopamine, 5×10^{-6} M	3	41.7±3.5 (9)	55.0±4.8 (9)	13.3	<0.05
	7	49.1±5.1 (6)	46.6±4.1 (6)	2.5	NS

Ethanol and piribedil were administered i.p. and body temperature monitored as described in the text and legend to Table 1. Dopamine-sensitive adenylate cyclase was measured as described in the text; basal values were similar to those in the legend of Fig. 1 and were not significantly different between ethanol-treated and control animals. All values represent mean ± s.e.m.; values in parentheses represent number of animals or, for adenylate cyclase activity, number of experiments.

*Values on any particular day were compared by the Student's *t* test.

after tolerance had developed did not prevent expression of tolerance¹⁸. Our previous data thus indicated that neurones other than those of the noradrenergic system would be responsible for the expression of tolerance to the hypothermic effects of ethanol. The postulated interaction between noradrenaline and dopamine-containing neurones¹⁹, in which activity in one system could exert a modulatory influence on the other system, would lend support to a hypothesis that dopaminergic systems may be involved in expression of tolerance to the hyperthermia produced by ethanol.

Although the time course of disappearance of decreased dopaminergic sensitivity seems to parallel the time course for disappearance of tolerance to ethanol, future studies should also evaluate the pattern of development of the alterations in dopamine receptor sensitivity, in order to more clearly define the possible relationship of such changes in the dopaminergic systems to the development of physical dependence on ethanol.

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Desensitisation does not selectively alter sodium channels

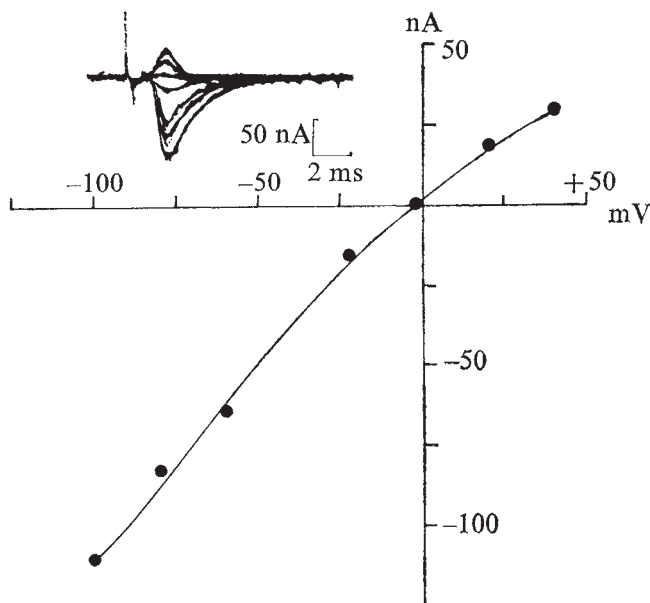
WHEN depolarising drugs are applied to the endplate region of skeletal muscle fibres, the postjunctional membrane undergoes a rapid increase in ionic conductance. This increased permeability, largely for Na^+ and K^+ , is slowly reversed if the agonist application is sustained. The gradual diminution of response to agonist is due to endplate desensitisation¹. Many factors influencing desensitisation have been described, but the molecular mechanisms responsible for the development of desensitisation remain unknown. Two fundamental types of mechanisms have been postulated to explain the phenomenon of desensitisation²⁻⁶. In the first, desensitisation is viewed as resulting from an alteration in the chemical receptor or binding site. The second type of mechanism suggests that desensitisation results from an alteration at some extrareceptor site such as that controlling the ionic channel in the endplate membrane. Kuba and Koketsu⁷ have shown that the reversal potential for acetylcholine was shifted to more negative values at desensitised endplates. They argued that this change was due to a selective decrease in G_{Na} and therefore supported the view that an extrareceptor mechanism was important in the development of desensitisation. In contrast Koester⁸ found no significant alteration in the reversal potential at desensitised endplates. In view of these conflicting observations we have re-investigated the question of whether there is or is not a selective alteration in end-

plate conductance during desensitisation. We report here that desensitisation does not selectively alter sodium channels.

All experiments were performed *in vitro* on sciatic nerve-sartorius muscle preparations from the frog, *Rana pipiens* maintained in a phosphate-buffered Ringers solution⁹ or a Tris-buffered, Na^+ -free, isotonic K^+ propionate solution⁹. For some experiments, the phosphate-buffered Ringers solution was made 2.3 times hypertonic by the addition of sucrose to minimise muscle contraction during carbachol application^{6,10}. In other experiments, neuromuscular transmission was depressed by the addition of curare ($2-3 \text{ mg l}^{-1}$) to the bathing solution to reduce the amplitude of the postjunctional conductance change. Endplate desensitisation was induced with carbachol. The carbachol was either added directly to the solution bathing the muscle preparation or was locally microperfused on to the endplate region of individual muscle fibres by hydrostatic pressure from an 85-100 μm diameter pipette placed within 50 μm of the recording and current-passing electrodes^{6,10}. Junctional regions of individual fibres were located visually by following nerve twigs to the last node of Ranvier using transmitted light and a magnification of 300. Conventional electrophysiological techniques were used to measure resting membrane potentials. The neurally-induced endplate current (EPC) and carbachol-induced currents were determined with point voltage-clamp techniques using a two microelectrode voltage clamp similar in principle to that of Takeuchi and Takeuchi¹¹ and in detail to that described by Gibbons and Fozzard¹².

The possibility that desensitisation results in a selective decrease in Na^+ conductance at the endplate membrane was evaluated by comparing the reversal potential for neurally-evoked EPCs of curarised preparations to those of partially-desensitised endplates. We reasoned that a selective decline in Na^+ conductance during desensitisation would shift the reversal potential to a more negative value. Curare was used so that non-desensitised EPCs had similar amplitudes to desensitised non-curarised EPCs. Individual fibres were voltage-clamped over the range -100 mV to $+40 \text{ mV}$, and the reversal potentials were determined by interpolation (Fig. 1). The reversal potential obtained from curarised ($2-3 \text{ mg l}^{-1}$) junctions ($E_r = -2.4 \pm 2.8$

Fig. 1 Example of the current-voltage relationship obtained at a desensitised endplate. The muscle from which this record was obtained was bathed in a phosphate-buffered Ringers solution containing 55 μM carbachol. The data were gathered after exposing the muscle to carbachol for 25 min. In this particular fibre the resting potential was -44 mV . The inset shows the current traces from which the current-voltage curve was plotted. An upward deflection indicates outward current and a downward deflection indicates inward current. The EPC reversal potential, determined by interpolation is -2 mV .



mV; mean $\pm$ s.e., $n = 9$) was similar to that obtained from 55- μ M carbachol-desensitised junctions ($E_r = -4.4 \pm 1.2$ mV; mean $\pm$ s.e., $n = 14$). This difference was not statistically significant ($P > 0.05$, Student's t test). These values of the reversal potential are comparable to those reported by others^{13,14}. Estimates of the reversal potential were obtained during a 20–110-min exposure to curare or a 10–80-min exposure to 55 μ M carbachol. In some carbachol-treated preparations there was a progressive decrease in the neurally-evoked EPC amplitude during this period whereas in other preparations the amplitude remained relatively constant during drug exposure. Nevertheless, the value of the reversal potential was similar in both instances. In the carbachol-treated fibres, the resting potential was initially low and progressively returned towards the pre-carbachol level with time⁶. This was taken as evidence for the continued development of desensitisation in these fibres.

If desensitisation selectively affects G_{Na} without influencing G_K , then carbachol-induced currents should not decay at E_{Na} where the EPC is primarily a potassium current. To test this possibility, the activation-desensitisation sequence was evaluated at E_K and E_{Na} . This was accomplished by monitoring the EPC produced by local microperfusion of 110 μ M carbachol on to individual end plates voltage-clamped at -100 mV or $+50$ mV, two values assumed to be reasonable approximations of E_K and E_{Na} , respectively. To minimise muscle contraction, these preparations were maintained in a hypertonic phosphate-buffered Ringers solution¹⁰. Microperfusion of 110 μ M carbachol on to the endplate region of these voltage-clamped fibres induced a transient current superimposed on the holding current. At both voltages, the carbachol-induced EPC developed with time to a peak of a few seconds, then slowly decayed toward the pre-carbachol level even though the carbachol application was continued (Fig. 2). The decay of the carbachol-induced EPC indicates that desensitisation occurred at both E_K and E_{Na} . We have used the time constant of current decline as an index of the time course of desensitisation. In these two examples, desensitisation developed more slowly in the fibre voltage-clamped at $+50$ mV ($\tau = 38.6$ s) than in the other fibre voltage-clamped at -100 mV ($\tau = 7.0$ s). Similar results were obtained from three other muscle preparations, one fibre from each preparation being voltage-clamped at -100 mV and another at $+50$ mV during carbachol application. We attribute the difference in desensitisation rates at these two voltages to the voltage dependence of desensitisation as described by Magazanik and Vyskocil⁴; hyperpolarisation accelerating and depolarisation slowing the process.

Fig. 2 Time course of endplate current produced by sustained microperfusion of 110 μ M carbachol. *a*, Response from an endplate voltage-clamped at $+50$ mV. *b*, Response from an endplate voltage-clamped at -100 mV. -100 mV and $+50$ mV were assumed to be reasonable approximations of E_K and E_{Na} , respectively. To minimise muscle contraction during carbachol microperfusion, these muscles were bathed in a phosphate-buffered Ringers solution made 2.3 times hypertonic by the addition of sucrose. Arrows indicate the onset of carbachol microperfusion. An upward deflection indicates outward current and a downward deflection indicates inward current.

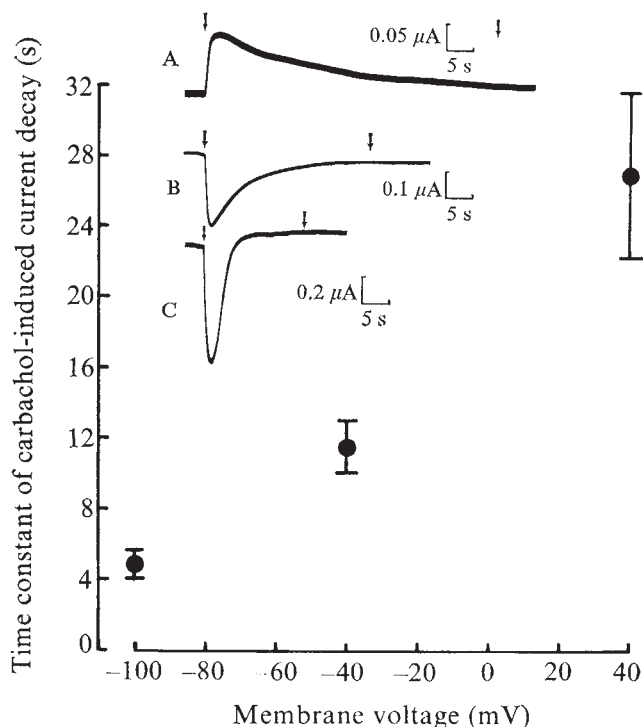
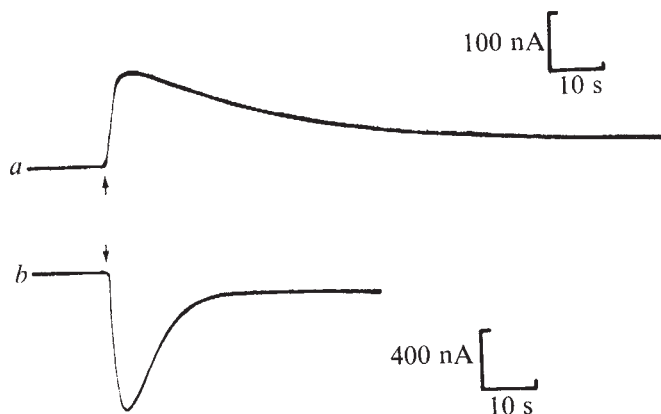


Fig. 3 Voltage dependence of desensitisation produced by 250 μ M carbachol in K^+ depolarised muscle fibres. These data were obtained from fibres bathed in a Tris-buffered, Na^+ -free, isotonic K^+ propionate solution. The time constant of carbachol-induced current decay during continued carbachol perfusion is plotted as a function of membrane potential. Each point represents the mean $\pm$ s.e. obtained from at least five fibres. The inset shows examples of 250 μ M carbachol-induced currents from three different fibres measured in voltage clamp conditions. The fibres were voltage-clamped to: $+40$ mV (*a*), -40 mV (*b*), and -100 mV (*c*), respectively. Arrows indicate the onset and termination of carbachol application. An upward deflection indicates outward current and a downward deflection indicates inward current.

Desensitisation occurs in K^+ -depolarised muscle preparations in the absence of extracellular sodium^{9,10}. In these conditions, the agonist-induced current is carried primarily by K^+ . We therefore evaluated desensitisation induced by local microperfusion of 250 μ M carbachol in voltage-clamped muscle fibres (V_c at either -100 mV, -40 mV, or $+40$ mV) maintained in a Na^+ -free, isotonic potassium propionate Ringers solution. Figure 3 indicates that the rate of desensitisation in these potassium depolarised fibres is voltage dependent, and the dependence is similar to that observed in polarised fibres (compare with Fig. 2). These observations indicate that the voltage dependency of desensitisation is maintained regardless of the ionic current-carrying species.

In agreement with Koester⁸, we find no difference in EPC reversal potential between curarised and partially desensitised endplates. The data presented here suggest there is no selective alteration in G_{Na} at desensitised endplates. Because the current-voltage relationship for the EPC is nonlinear^{13,14}, estimates of the reversal potential obtained by extrapolation can be misleading. Although Kuba and Koketsu's⁷ plots show little evidence of nonlinearity, this may account, in part, for the conflict between conclusions drawn previously by Kuba and Koketsu⁷ and with those of Koester⁸ and the present report. It should be noted that the experimental conditions used in this study and by Koester⁸ are substantially different from those of Kuba and Koketsu⁷. In the former desensitisation was induced by applying carbachol to the bathing solution whereas in the latter desensitisation was produced by iontophoretic application of acetylcholine. Consequently, the different results may be due to different experimental conditions. But, in agreement with the data presented here, Manalis and Werman¹⁵, using experimental conditions similar to those of Kuba and Koketsu⁷, have demonstrated by interpolation that desensitisation does

not alter the acetylcholine reversal potential. In summary, our results show that desensitisation develops whether the endplate current is carried mainly by Na^+ and K^+ or by Na^+ and K^+ individually, and that differences in time course of desensitisation apparently are due to the voltage dependence of the desensitisation process⁴.

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Significance of extensive 'leaky' cell junctions in the avian salt gland

CURRENT models for hypertonic sodium secretion by salt glands assume there are physiologically tight junctions between the principal cells of the secretory tubules and that these cells actively secrete sodium across the cell membrane at the lumen in a one-stage process¹. Our recent data require consideration of a new two-stage hypothesis concerning the secretory mechanism. Our freeze–fracture studies demonstrate that these cell junctions (*zonulae occludentes*) in the nasal salt gland of the herring gull (Fig. 1) should be classified as 'very leaky'². Also, the extraordinary conformation of the apices of the principal cells within the epithelium makes these cell junctions most extensive (Fig. 2). Therefore, we propose that the salt glands of vertebrates produce their hypertonic effluent from the primary isotonic secretion of the peripheral cells at the ends of the secretory tubules, followed by the secondary reabsorption of water

Fig. 1 Freeze–fracture image through the luminal membranes (P-faces) of three adjacent apical processes of the principal cells in the salt gland of a herring gull. It demonstrates clearly that the *zonulae occludentes* (arrowheads) are single-stranded. Circular profiles of the bases of several microvilli and a small part of the lumen filled with finely-textured ice (lower right) are also shown. Karnovsky fixation, Balzers freeze–fracture preparation, Zeiss 10B electron microscope, $\times 51,360$.

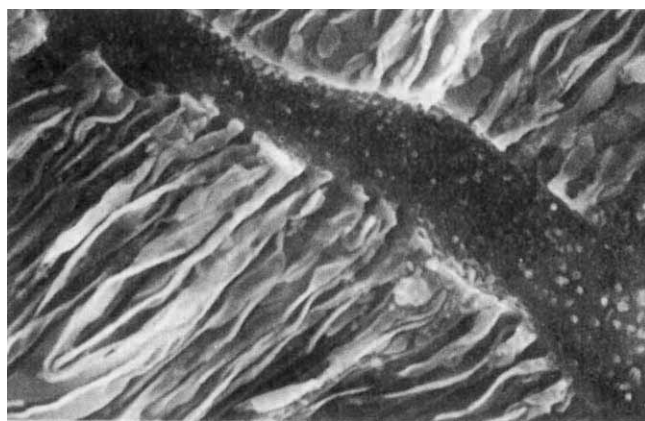
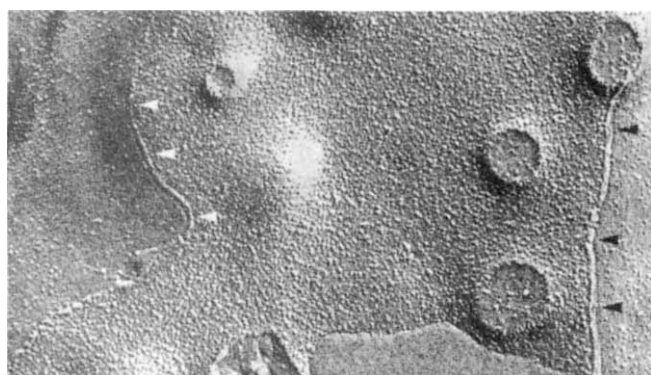


Fig. 2 The lumen of a secretory tubule from the nasal salt gland of a herring gull traverses diagonally from upper left to lower right through this scanning electron micrograph. Knoblike microvilli dot the surface of the lumen. The intermeshing apical processes of the principal cells form the wall of the lumen. Folded lateral surfaces of the principal cells (lower left and upper right) delimit long intercellular channels extending away from the luminal cell junctions. The complementary surfaces of overlying principal cells were pulled purposely out of their positions during specimen preparation. Karnovsky fixation, osmium-thiocarbohydrazide coating, critical point drying with CO_2 , recorded with an AMR-1000 scanning electron microscope, $\times 5,928$.

through the leaky junctions of the principal cells and not simply by the extrusion of Na^+ across the luminal membranes of the principal cells. This new hypothesis is consistent with the accepted theories of sweat gland and salivary gland function, except that in the salt gland the second stage after isotonic secretion is water absorption, rather than salt reabsorption.

Within the salt gland the lobules contain branched, blind-ended secretory tubules (Fig. 3) that radiate from a central excretory canal³. The diameter of the lobules and the length of the secretory tubules are both directly correlated with the concentration of Na^+ in the effluent of the gland⁴. Also, the size and height of the principal cells that predominate in these tubules increase progressively along a tubule from the periphery toward the centre of each lobule⁵. Within the cytoplasm of these cells large, abundant mitochondria are closely associated with the lateral membranes along the intercellular channels⁶. In addition, cytochemical^{7–10} and autoradiographic¹¹ studies show that the enzyme system responsible for sodium transport is found in these lateral plasma membranes and not in the luminal membranes of these cells.

In contrast, the cuboidal peripheral cells (Fig. 3), situated in the blind end of each tubule are relatively small. They resemble closely the 'clear' secretory cells that are found in the secretory tubules of eccrine sweat glands, and have cytochemical^{12,13} and ultrastructural⁶ characteristics that are quite different from the principal cells.

Freeze–fracture images (Fig. 1) through the plasma membranes at the luminal interface of principal cells show that the *zonulae occludentes* consist of a single strand. Thus, by comparison with other epithelia², this junction must be classified as very leaky. This classification is substantiated also by earlier tracer studies on salt glands^{14,15}.

The strikingly complex plicated lateral surfaces of the principal cells in the secretory tubules of the salt gland of the herring gull is revealed by the scanning electron microscope (Fig. 2). Folds of these membranes form the boundaries of long intercellular channels that extend from the apical cell junctions to the extracellular space at the base of the epithelium. In addition, the complicated intermeshing at the lumen of the apical ends of the principal cells⁶, means that the leaky cell junctions are unusually extensive.

Figure 3 shows our conception of how a secretory tubule

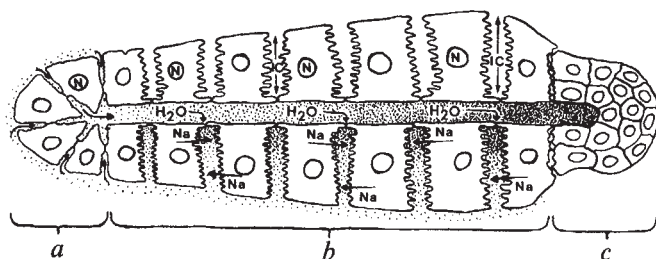


Fig. 3 In this diagram of a single secretory tubule in a salt gland, the number of cell junctions as well as the complexity of the intercellular channels are greatly reduced, and the diameter of the lumen of the tubule is much enlarged. The terminal segment (a) of the tubule is composed of small peripheral cells; the remainder of the tubule (b) is lined by principal cells. The central excretory canal (c) is continuous with an excretory duct (not shown). The intercellular channels (IC) are closed at their luminal ends by leaky cell junctions. The density of the stipple in the lumen and in the intercellular channels indicates the concentration of Na^+ .

produces a hypertonic secretion. Isotonic fluid secreted by the peripheral cells at the terminal cul-de-sacs (a) flows along the lumen of the tubule towards the central canal (c). As this secretion moves over the apices of the principal cells that line most of the tubule (b), water is absorbed from this fluid through the leaky junctions between the principal cells and into the intercellular channels where a standing gradient¹⁶ of Na^+ is maintained by the sodium pumps in the lateral membranes of the cells. Na^+ ions are most concentrated at those ends of the channels near the leaky cell junctions that border the lumen of the tubule and are least concentrated at the bases of the channels where they are open to the subjacent extracellular space. In maintaining the standing gradient, NaCl is recycled by the principal cells. NaCl enters the cells from the blood across the basal membrane, is pumped out the lateral membrane into the intercellular channels, and then returns to the blood. The NaCl moves from the blood into the cell, passing down a concentration gradient created by the lateral extrusion pumps. As a consequence of this standing gradient, water flows from the lumen into the intercellular channels. The concentration of Na^+ in the fluid that remains in the lumen of the tubule will approach the maximum of the standing gradient that is achieved in the intercellular channels. Accordingly, the height of the principal cells, and hence the length of the intercellular channels, along with the level of operation of the Na^+ pumps, produce also a standing gradient within the lumen of the tubule and affect the Na^+ concentration of the final effluent. Simply stated, the principal cells do not secrete the hypertonic effluent of the glands. Instead, water is passively reabsorbed through the leaky cell junctions from the isotonic fluid that is secreted by the peripheral cells. This differs from the original form of the standing gradient hypothesis¹⁶ which postulated water flow across the apical and lateral cell membranes.

This hypothesis requires that NaCl extruded laterally into the intercellular spaces is recycled NaCl , and does not come from the lumen, also that the plasma membranes of the principal cells must be relatively impermeable to water. This latter requirement is not unique, since cells with similar properties are found, for example, within the loop of Henlé in the mammalian kidney. It is also consistent with experimental data on intercellular osmolarity in salt glands^{17,18}. In addition, extracellular coats that are detectable at the surface of the principal cells^{14, 19} might also affect the permeability of the membrane.

Our hypothesis solves the dilemma of the orientation of the sodium pumps. Localisation of the sodium pumps along the basal and lateral membranes⁷⁻¹¹ required under earlier hypotheses¹ that Na^+ be pumped into the cells, an unprecedented orientation. In this new hypothesis, the sodium pumps have

their usual outwards orientation and still have a role in hypertonic secretion.

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Composition of sarcoplasmic reticulum *in situ* by electron probe X-ray microanalysis

EXCITATION-contraction (E-C) coupling in striated muscle^{1,2} involves depolarisation of the surface membrane that propagates through invaginations of the surface membrane, the transverse tubular (T) system, to the interior of the fibre. Stored Ca is then released from the adjacent sarcoplasmic reticulum (SR), and activates the contractile proteins. The mechanism of release of Ca from the SR is not definitely known, although it is thought to be triggered by the T-tubule action potential either through electrical coupling, possibly involving voltage dependent charge movement³⁻⁵ across the T-SR foot processes⁶⁻⁷ and depolarisation of the SR^{2,8,9} and/or a chemical coupling involving a Ca induced Ca release⁹⁻¹². Depolarisation of the sarcoplasmic reticulum in skinned muscle fibres by Cl induces Ca release². The evaluation of the various models of E-C coupling requires some knowledge of whether the ionic distribution across the SR membrane can give rise to a potential and, specifically, whether the content of the SR resembles that of the extracellular or of the intracellular fluid. So, to measure the ionic composition of individual elements of the sarcoplasmic reticulum *in situ*, we used electron probe X-ray microanalysis of frozen-dried thin section of a fast striated muscle. The data we report here show that neither Cl nor Na are compartmentalised in the SR, and that their concentrations in the SR (on a dry weight basis) do not differ from those in the surrounding cytoplasm. Ca was found to be sequestered in the sarcoplasmic reticulum confirming other studies (refs 13, 14 and see refs 1, 2 for reviews). These findings (albeit on a dry weight basis) do not support the existence of a Cl or Na potential across the SR membrane in resting muscle.

The swimbladder muscle of the toadfish *Opsanus tau* is a very fast twitch muscle with a time to peak contraction of 5-8 ms and an extraordinary development of the sarcoplasmic reticulum¹⁵. The extent of the SR (approximately 30% of fibre volume, C. Franzini-Armstrong, personal communication) and its very regular arrangement of two triads per A band (Fig. 1) make it very suitable for viewing in unfixed frozen-dried thin sections. The muscles were shot into supercooled Freon 22, sectioned at -130°C and

Table 1 Elemental concentrations of sarcoplasmic reticulum and cytoplasm of toadfish swimbladder determined by paired 'small spot' electron probe analysis (mmol per kg dry weight)

Region analysed	Na	P	S	Cl	K	Ca	Mg
Cytoplasm (between SR)	30 ± 4.3	275 ± 2.0	335 ± 2.0	47 ± 1.1	510 ± 2.7	4 ± 0.8	47 ± 1.9
Sarcoplasmic reticulum	34 ± 4.2	443 ± 2.5	362 ± 2.0	42 ± 1.0	589 ± 2.8	77 ± 0.9	40 ± 1.8

Values are means ± s.e.m., $n = 25$ pairs. We used a Philips EM 301 transmission electron microscope with a goniometer stage modified to accept a 30-mm² (20° specimen to detector angle, 0.067 sr solid angle, 160 eV resolution) Kevex Si(Li) X-ray detector interfaced with a Kevex 5100 multichannel analyser and a Tracor Norther NS 880 computer and TN 1,000 magnetic tape recorder. A modified Philips liquid nitrogen cooled holder, operated at -100 to -110 °C, was used to minimise contamination and mass loss. The detailed characteristics of the system have been described previously¹⁹. Some analyses were done with the Philips EM 400 operated with an LaB₆ source and interfaced as above. The better vacuum (2×10^{-7} torr) allowed us to obtain longer counts without contamination and the higher brightness of the LaB₆ source yielded a higher count rate. The probe diameters were 50–100 nm. The SR was probed in regions adjacent to the T-tubules where these could be identified. The regions of the cytoplasm analysed were within 200 nm of their respective SR pairs. The measurement of elemental concentrations is based on the fact, pointed out by Hall¹⁸, that for elements of atomic number $Z \geq 11$ in concentrations of less than 1 mol per kg, the characteristic peak/X-ray continuum ratio is linearly related to the concentration/dry mass. The peak counts in the unknown peak and its error of measurement are computed together with the counts in the continuum band 1.34–1.64 keV. The basic equation for quantitation is $C_x = (I_x/I_b)\chi W_x$, where C_x is the concentration of element x , I_x is the number of characteristic peak counts for that element, I_b is the number of continuum counts and W_x is a quantitation parameter relating concentration to peak/background ratio and is obtained from standards. W_x includes the effects of ionisation cross sections, fluorescence yield and detector efficiency for each specific element.

The X-ray spectrum obtained in a transmission electron optical column from a thin section includes the characteristic peaks due to elements $Z \geq 11$ present in the specimen and the associated continuum largely arising from the organic matrix. It also contains instrumental peaks (the largest one being the Cu signal $K_\alpha = 8.047$ keV) from the specimen grid and holder, with an associated extraneous continuum, and low energy noise probably due to electrons reaching the detector. These extraneous signals and the associated continuum can be measured by collecting the spectrum generated with the beam passing through an empty grid hole, and are subtracted by the computer routine as is the low energy noise¹⁹.

freeze-dried as reported previously¹⁶. Frozen-dried thin sections of unfixed muscle are shown in Fig. 2 and can be compared with a conventionally glutaraldehyde-osmium fixed section of the same muscle shown in Fig. 1. The junctional gap between the T-tubule and terminal cisterna^{6,7} is also present in the unfixed frozen sections, but the T-tubule is not as flattened as in fixed material (Fig. 2b). The longitudinal SR is narrower in the cryo sections and has a laminated appearance, suggesting that measurements in conventionally fixed material may give rise to overestimates of the *in vivo* SR volume. A collapsed longitudinal SR has been previously observed in frog striated muscles fixed in the presence of polyvalent cations¹⁷ and in freeze-fracture replicas of unfixed muscle (Franzini-Armstrong, Reese and Heuser, personal communication).

The concentrations of Na, P, S, Cl, K, Ca and Mg in, respectively, the SR or the adjacent cytoplasm (25 paired analyses with 50–100 nm probes) are shown in Table 1. Because the quantitation is based on the ratio of the characteristic peak to the

X-ray continuum counts^{18,19} from frozen-dried material, the data are expressed as mmol per kg dry weight. The mean Ca concentration in the SR of resting muscle is 77 ± 0.9 (s.e.m.) mmol per

Fig. 2 Longitudinal cryo sections from toadfish swimbladder muscle. The muscle mounted on a low mass stainless steel mesh holder was frozen by shooting it into supercooled (-164 ± 2 °C) Freon 22 and sectioned at -130 °C in a modified LKB cryokit. The sections were picked up dry onto Cu grids with carbon foils, dried at $< 10^{-6}$ torr overnight, carbon coated and stored dessicated. This section was stained with osmium vapour under vacuum to enhance contrast. Electron probe X-ray microanalyses were generally done on similar but unstained material. A detailed description of the freezing and cryoultramicrotomy is given elsewhere¹⁶. T-tubules are indicated by arrows. Circles indicate the approximate probe size used for the analyses summarised in Table 1. *a*, $\times 39,960$. *b*, $\times 53,680$.

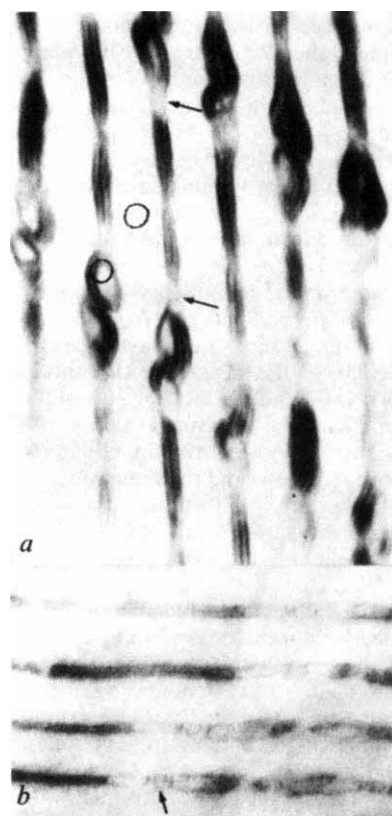
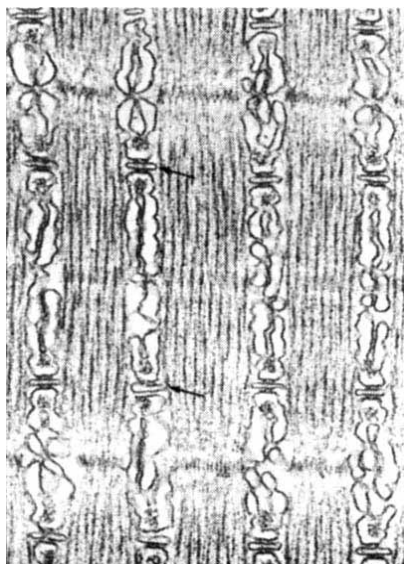


Fig. 1 Longitudinal section from a toadfish swimbladder muscle fixed in glutaraldehyde, post fixed in osmium and embedded. T-tubules indicated by arrows. ($\times 41,580$).



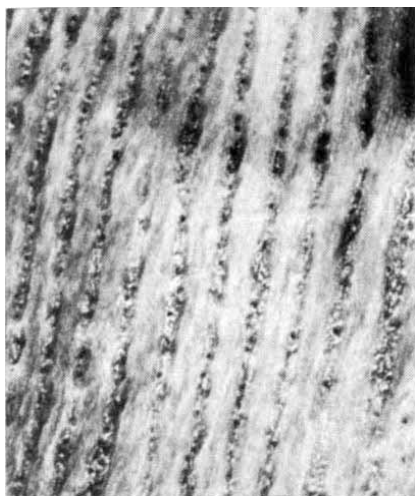


Fig. 3 Longitudinal cryo section from a damaged toadfish swim-bladder muscle fibre. Unstained. The sarcoplasmic reticulum is loaded with electron dense granules. ($\times 4,650$.)

kg. Because much of the phosphorus in the SR region (Table 1) could be within membrane phospholipids, these experiments do not indicate whether P (phosphate) is a counterion for Ca. The Ca concentration in the regions of cytoplasm between the SR shown in Table 1 was higher than in frog muscle¹⁶. This may reflect some overlap of obliquely oriented SR in the narrow segments of cytoplasm analysed (Fig. 2) and/or Ca binding to the parvalbumins in fish muscle²⁰. Recent studies, however, indicate that the parvalbumin contents of frog and fish muscle are similar²⁴.

The Na and the Cl concentrations in, respectively, the SR and adjacent cytoplasm were not significantly different. This finding does not support the existence of a Na or Cl potential across the SR membrane, however, electron probe analysis of freeze-dried cryo sections measures total (bound and free) ion contents rather than activities. The greater (by approximately 80 mmol per kg) concentration of K detected over the SR than over the cytoplasm is similar to our observations on frog striated muscle SR¹⁶ and could arise from differences in hydration between the two regions or from K binding to the SR and its acidic proteins^{21,22}.

The results of analyses of 1–6 μ m diameter areas that included both SR and cytoplasm (in 13 fibres from 4 toadfish) were: (mmol per kg dry weight $\pm$ s.e.m.): Na, 42 ± 7.2 ; P, 360 ± 4.3 ; Cl, 56 ± 1.9 ; K, 539 ± 5.1 ; Ca, 26 ± 1.6 ; Mg, 36 ± 3.0 . The relatively high (26 ± 1.6 mmol per kg dry weight) Ca content detectable with the probe parameters used is primarily due to the large volume occupied by the SR in the swimbladder muscle. The other elemental concentrations obtained with these large diameter probes also substantiate the absence of 'extracellular' ion compartmentalisation in the SR (apart from Ca), shown in Table 1. The bulk NaCl content that would have been expected (277 mmol per kg dry weight) on the basis of an SR containing extracellular fluid (231 mM NaCl) and constituting 30% of fibre volume is very much higher than the measured values (Na, 42 ± 7.2 ; Cl, 56 ± 1.9 mmol per kg dry weight). Minor differences between these Na and Cl concentrations and those measured with small probes (Table 1) were due to fibre-to-fibre variations. The Na and Cl concentrations measured with large (1–6 μ m) or small (50–100 nm) probes in the same fibre were not significantly different.

In one damaged fibre not included in the tabulated results, the SR was loaded with granules containing Ca, Mg and P (Fig. 3). Analyses of this fibre with large diameter probes showed Ca concentrations of 76 ± 3.5 mmol per kg dry weight indicating a threefold increase in total fibre Ca. The maximum Ca capacity of the SR is estimated to be three to four times greater than its physiological Ca content^{2,23}.

The results of this study are in agreement with our findings on frog striated muscle¹⁶, that the Cl concentration of the SR does not

approach extracellular levels. In addition, the results on toadfish SR show a similar absence of compartmentalised Na; this excludes the possibility that the relatively low Cl concentration in the SR is due to Donnan exclusion by acidic proteins. We conclude that the T-SR junction in resting muscle is not freely permeable to Na or Cl.

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Myoblast myosin phosphorylation is a prerequisite for actin-activation

CYTOPLASMIC myosins are found in most non-muscle cellular systems studied to date¹. In vertebrates these myosins are composed of two heavy chains of 200,000 molecular weight (MW) and four light chains, two each of 20,000 and 15,000 MW. A number of cytoplasmic myosins, as well as skeletal, smooth, and cardiac muscle myosin, can be enzymatically phosphorylated^{2–6}. That is, those light chains of MWs 18,000–20,000, the so-called P-light chains⁶, can undergo phosphorylation catalysed by a myosin light chain kinase^{7,8}. In this reaction the terminal phosphate of ATP is transferred to a specific amino acid residue on the myosin P-light chain, up to one mole of phosphate per mole of light chain^{9,10}. A myosin light chain phosphatase that catalyses the removal of the covalently-linked phosphate group from the P-light chain, returns the myosin to the non-phosphorylated state^{5,11}. Phosphorylation of human platelet myosin⁴, guinea pig vas deferens³ and chicken gizzard^{12,13} smooth muscle myosin results in an increase in the actin-activated ATPase activities of these myosins. Although the phosphorylation of the P-light chain of rabbit skeletal muscle myosin has been described¹⁰, no functional change in this myosin's activity due to phosphorylation has been demonstrated¹¹. To study the role of myosin phosphorylation during muscle cell differentiation, we investigated precursor cells to skeletal muscle, namely proliferative myoblasts. The Yaffe

L-5 810 line of cloned proliferative rat myoblasts is an actively dividing cell line in culture which under the appropriate conditions can form myofibrils. We now report: (1) that myosin isolated from proliferative myoblasts contains light chains of 20,000 and 15,000 MW, (2) the 20,000 MW light chain can be phosphorylated by an endogenous myosin light chain kinase¹⁴, (3) the proliferative myoblast myosin light chain kinase does not require calcium ions for activity, and (4) phosphorylation is a pre-requisite for actin activation of the myosin's ATPase activity.

Myosin and the myosin light chain kinase were isolated from 3 g of proliferative rat myoblasts by extraction at high ionic strength (0.5 M KCl, 15 mM Tris-HCl (pH 7.5), 10 mM $\text{Na}_2\text{P}_2\text{O}_7$, 10 mM dithiothreitol (DTT), 1 mM EDTA), followed by dialysis against low salt (0.05 M KCl, 10 mM Tris-HCl (pH 7.5), 5 mM DTT, 0.1 mM EDTA) to form a precipitate and sedimented at 48,000g for 10 min. The pellet was suspended in 3 ml of 0.5 M KCl, 15 mM Tris-HCl (pH 7.5), 1.0 mM EDTA, 2.5 mM DTT (protein concentration 5–10 mg ml⁻¹) and then made 10 mM in MgATP and fractionated with ammonium sulphate.

When the 35–55% saturation ammonium sulphate fraction was dialysed to remove the ammonium sulphate and then

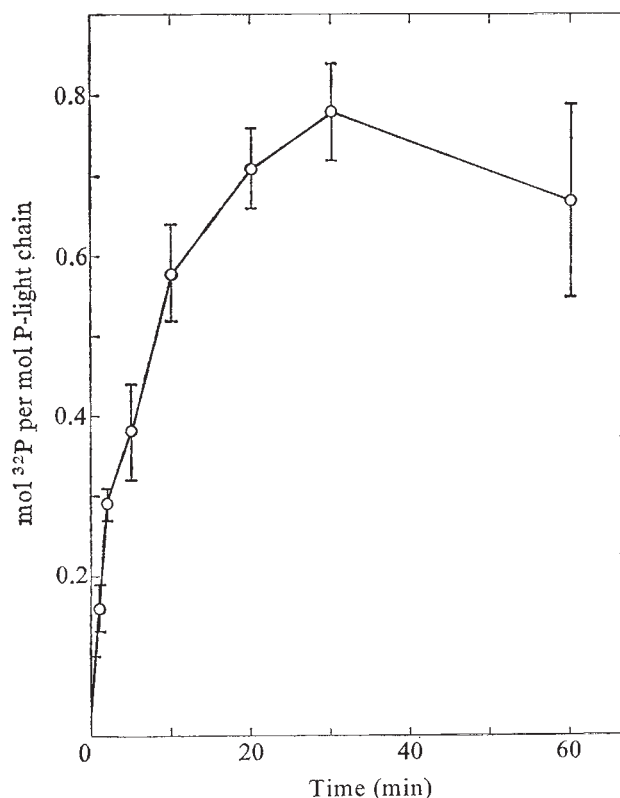


Fig. 1 Time course of ³²P incorporation into the proliferative myoblast myosin P-light chain by the endogenous myosin light chain kinase. The 35–55% saturation $(\text{NH}_4)_2\text{SO}_4$ fraction of actomyosin was dialysed against 0.5 M KCl, 15 mM Tris-HCl (pH 7.5), 2.5 mM dithiothreitol (DTT), 1 mM EDTA and incubated in the phosphorylation conditions: 0.2 M KCl, 30 mM Tris-HCl (pH 7.5), 12.5 mM MgCl_2 , 5 mM DTT, 0.2 mM CaCl_2 , and 0.1 mM ATP. The ATP was premixed with $\text{Na}_4\gamma\text{-ATP}^{32}\text{P}$ (30 Ci mmol⁻¹) so as to give 200,000 d.p.m. in 5 μl of the final mixture (0.4 ml). The reaction was stopped by the addition of SDS to a final concentration of 1% and 0.05% $\text{Na}_2\text{P}_2\text{O}_7$. These samples were then dialysed to equilibrium against 0.3 M, 0.2 M, 0.1 M NaCl, and finally deionised water to remove the excess ATP^{32}P . 1% SDS–7.5% polyacrylamide gel electrophoresis was carried out as described previously¹⁶ with the exception that diallyltartardiamide (BioRad) was substituted for bis-acrylamide in the same molar ratio to the acrylamide¹⁷. The P-light chain was cut out of the gel and dissolved with 2% periodic acid and then counted in ACS (Amersham/Searle) scintillant. The data are for four determinations from three different preparations $\pm$ s.d.

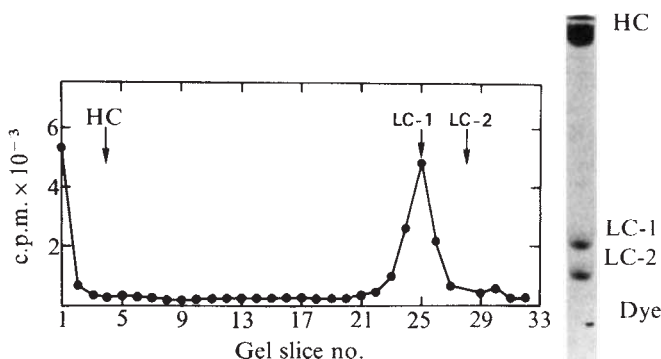


Fig. 2 Incorporation of ³²P into column-purified proliferative myoblast myosin with column-purified proliferative myoblast light chain kinase. Unphosphorylated myoblast actomyosin (35–55% $(\text{NH}_4)_2\text{SO}_4$ fraction) was made 10 mM in MgATP immediately before fractionation on a Sepharose 4B column (1.5 × 90 cm), which was equilibrated and eluted with 0.5 M KCl, 15 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2.5 mM DTT. The 4 °C temperature, high ionic strength (0.5 M KCl), and speed of application of the sample to the column were necessary to inhibit phosphorylation in the unphosphorylated actomyosin. The myosin and myosin light chain kinase eluted as distinct peaks, were pooled individually, and then aliquots were mixed in the phosphorylation conditions and processed for gel electrophoresis as in Fig. 1, except that 5 μl of the final mixture gave 40,000 d.p.m. There were 3 μg of the P-light chain on the gel. The myosin light chain kinase fraction was purified only partially by gel filtration. But, only catalytic amounts of this fraction were used for the phosphorylations, as can be discerned by this gel, since no band at 80,000 MW characteristic of either the skeletal⁷ or platelet⁸ kinase can be detected. In similar experiments, the samples were treated with RNase, which eliminated the counts at the top of the gel, but did not change the incorporation into the 20,000 MW light chain. HC, myosin heavy chain (200,000 MW); LC-1, myosin light chain one, the P-light chain (20,000 MW); LC-2, myosin light chain two (15,000 MW).

incubated with $\text{MgATP}^{32}\text{P}$, incorporation of ³²P was observed in the fraction as is shown in Fig. 1. Aliquots of the phosphorylated fractions at different times of incorporation were electrophoresed in 1% sodium dodecylsulphate (SDS)–7.5% polyacrylamide gels, stained for protein, and the entire gel was cut into slices which were then counted for ³²P. As Fig. 1 shows, within 30 min 0.8 mol of ³²P were incorporated per mol of P-light chain. Thus it was determined that an endogenous myosin light chain kinase was present in the proliferative myoblast actomyosin fraction.

Gel filtration of the 35–55% ammonium sulphate fraction on Sepharose 4B resulted in separation of purified myosin from the kinase (see refs 5, 3 for similar results with platelet and smooth muscle actomyosin). An SDS–polyacrylamide gel of such a pooled myosin fraction is shown in Fig. 2. The apparent MWs in SDS of the light chains were 20,000 (LC-1) and 15,000 (LC-2), similar to other proliferative myoblast myosin¹⁵. When this purified myosin fraction was incubated with the myosin light chain kinase fraction from the same column in the phosphorylation conditions (see Fig. 1 legend) and then electrophoresed on an SDS–polyacrylamide gel, incorporation of ³²P was seen only in the 20,000 MW light chain, the P-light chain. The amount of incorporation depicted in this figure is 0.76 mol of ³²P per mol of P-light chain. As controls, these two fractions were incubated separately in the phosphorylation conditions and subjected to gel electrophoresis as in Fig. 2. No incorporation was seen in either of these fractions.

A major difference between the kinase present in rat proliferative myoblasts and adult rat thigh muscle is shown in Table 1. Whereas the adult rat muscle kinase requires Ca^{2+} for its activity, the myoblast kinase does not. In this respect the myoblast myosin light chain kinase is of the cytoplasmic type, similar to those of human platelet⁸ and mouse astrocytic neuroglial myosin². The rat thigh muscle kinase is similar to that of rabbit skeletal muscle in requiring Ca^{2+} for activity⁷.

The effect of phosphorylation on the specific ATPase

Table 1 Effect of calcium on the activity of the myosin light chain kinase

Kinase	Substrate	mol ³² P incorporated per mol P-light chain	
		+Ca ²⁺	-Ca ²⁺
Proliferative myoblast	Myoblast myosin	0.75	0.81
	Skeletal myosin light chains	0.91	0.86
Rat thigh muscle	Skeletal myosin light chains	0.93	0.01

Either proliferative rat myoblast or adult rat thigh muscle myosin light chain kinase was column-purified as described in Fig. 2. These kinases were then incubated for 60 min under the phosphorylation conditions (1 Ca²⁺) as in Fig. 1 or in the presence of 2 mM EGTA (-Ca²⁺) with native myoblast myosin or myosin light chains from rabbit skeletal muscle isolated from a 35–55% (NH₄)₂SO₄ fraction according to the method of Perrie and Perry¹⁸. All the values are calculated from 1% SDS–10% polyacrylamide gels as described in Fig. 1.

activities of phosphorylated and unphosphorylated proliferative myoblast myosin is shown in Table 2. To obtain phosphorylated and unphosphorylated myosin the 35–55% ammonium sulphate fraction was divided in half and was either phosphorylated or incubated without ATP and then chromatographed on a Sepharose 4B column. The purified myosin was pooled and the ionic strength was lowered to 20 mM KCl by dialysis. No actin activation of the myosin ATPase activity at low ionic strength (0.017 M KCl) can be demonstrated for the unphosphorylated myosin (Table 2). But a 9–12-fold increase in the myosin ATPase with actin is seen for the phosphorylated myosin. No effect of phosphorylation was seen on the high salt (0.5 M KCl) ATPase activity of myosin alone. Therefore, phosphorylation specifically affects the actin interaction with myosin.

Table 2 also demonstrates that even though the actin concentration is tenfold greater than the myosin concentration (mg per mg), there is no increase in the actin-activated ATPase activity on the addition of actin to unphosphorylated myosin. Using platelet myosin⁴, a small amount of actin-activation of the myosin ATPase activity in the control (unphosphorylated) sample has been observed. In that work, it was unclear whether

Table 2 The effect of phosphorylation on proliferative myoblast myosin ATPase activity

	Actin-activated (+Ca ²⁺)*	
	-Actin	+Actin
Phosphorylated	0.015	0.175
Unphosphorylated	0.018	0.019
	Actin-activated (-Ca ²⁺)	
	-Actin	+Actin
Phosphorylated	0.019	0.181
Unphosphorylated	0.020	0.022
	Myosin alone†	
	K ⁺ -EDTA	Ca ²⁺
Phosphorylated	1.31	0.218
Unphosphorylated	1.28	0.225

Specific activity is expressed as $\mu\text{mol Pi}$ per mg myosin per min at 37 °C. The standard deviations of these values are less than or equal to 15%. Actomyosin fractions of proliferative rat myoblast myosin (see Fig. 1) were divided in half. One half was phosphorylated and the other half was incubated in the phosphorylation conditions without ATP. These fractions were then column-purified (see Fig. 2). These fractions gave similar electrophoretic patterns to those of Fig. 2. The data presented are from six determinations on four different preparations. Inorganic phosphate liberation was measured by a modification of the method of Martin and Doty^{4,20}. In some experiments phosphate release was measured as ³²P released from AT³²P using the same method²⁰, but without the development of colour. Protein concentrations were estimated by the method of Lowry *et al.*²¹ using bovine serum albumin as a standard.

*Assay conditions: 17 mM KCl, 10 mM Tris-HCl (pH 7.4), 1 mM ATP, 1.4 mM MgCl₂, 0.1 mM CaCl₂ (1 Ca²⁺) or 1 mM EGTA (-Ca²⁺). Myoblast myosin 0.04–0.10 mg ml⁻¹, rabbit skeletal muscle actin¹⁹, 0.4–1.0 mg ml⁻¹.

†Assay conditions: 0.5 M KCl, 20 mM Tris-HCl (pH 7.4), 2 mM ATP, and either 2 mM EDTA or 10 mM CaCl₂. Myoblast myosin, 0.04–0.07 mg ml⁻¹.

this actin activation was due to a small amount of phosphorylation or was a property of the non-phosphorylated myosin. In this report we show that before phosphorylation no actin activation of the myosin ATPase activity occurs (see Table 2) indicating that in the proliferative myoblasts, myosin phosphorylation is a prerequisite for actin activation. Further Table 2 shows that there is no difference in the actin-activated ATPase activity of phosphorylated myosin measured in the presence of Ca²⁺ and EGTA. This is similar to the results reported for phosphorylated platelet myosin⁵, but differs from the findings for vas deferens myosin³.

Myosin phosphorylation has been demonstrated previously to increase the actin-activated ATPase activity of platelet and smooth muscle myosin. The extension of this regulatory system to proliferative myoblasts, as well as the identification of myosin light chain kinase in astrocytic neuroglial cells² and HeLa cells (J. A. Trotter, S.P.S. and R.S.A., unpublished) suggests that myosin phosphorylation is a general regulatory mechanism controlling the actin-myosin interaction in both non-muscle and smooth muscle cells.

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A synthetic polypeptide with antifreeze activity

Much information has been gathered in recent years on the so-called 'antifreeze' proteins which lower the freezing point of the serum of certain marine fishes living in sub-zero water temperatures^{1–4}. The proteins from the Antarctic fish *Trematomus borchgrevinki* are glycoproteins with a repeating alanyl-alanyl-threonyl tripeptide sequence, the threonyl residue being linked to a disaccharide^{1,2}. In contrast, the antifreeze protein from the winter flounder *Pseudopleuronectes americanus* in the North American Atlantic coastal region is made up of eight amino acids with no apparent repeating sequence of the residues and no sugar moiety (ref. 4 and unpublished work of C. L. Hew, C. C. Yip & G. Fletcher). The antifreeze activity of these proteins is not compatible with the known colligative properties of solutes in solution and the mechanism of their action is not yet fully understood. But a common feature of both types of the antifreeze proteins is the preponderance of alanine which accounts

for over 60% of the total amino residues. This fact, together with the absence of the carbohydrate in the protein from the winter flounder, prompted us to attempt the synthesis of polypeptide analogues having comparable proportions of alanine in them along with suitable other amino acids. As a first step, we made use of the lack of any obvious periodicity in the distribution of the alanyl residues in the flounder's protein and attempted the synthesis of a random copolypeptide containing about 65 mol % of alanine and 35 mol % of aspartic acid. The choice of aspartic acid was made on the basis of its being the next major amino acid in the flounder's protein^{3,4} and on the expectation that its polar character will help the water-solubility of the alanine-rich copolypeptide, as in other studies on alanine-containing random copolymers. In addition, Duman and DeVries⁴ have earlier indicated the involvement of carboxyl groups on the antifreeze activity by chemical modification studies. We report here the synthesis of this polypeptide and show that it possesses antifreeze activity.

The copolypeptide was synthesised by first polymerising the *N*-carboxyanhydrides of L-alanine and β -benzyl L-aspartic acid in the molar ratio of 65:35 respectively in dioxane with triethylamine as the initiator; the β -benzyl group of aspartic acid was then removed by using either anhydrous hydrogen bromide⁶ or hydrogenfluoride-pyridine⁷. The products from both these procedures were very similar in their properties including the antifreeze activity.

Table 1 Properties of the synthetic polypeptide

Sample no.	Composition (mol %)*		Molecular weight†	α -helical content‡
	Alanine	Aspartic acid		
SAF-I§	65.5	34.5	45,000–70,000	15
SAF-II¶	64.8	35.2	50,000–70,000	20

*Determined on a Beckman Model 121M amino acid analyser after 24–48 h of hydrolysis in 12 M HCl at 110 °C. The compositions of the polypeptides before deblocking the side-chain benzyl group, were almost identical to those of the final products.

†Estimated using a Sephadex G-75 column calibrated with protein markers.

‡Estimated on the basis of the circular dichroism data as before⁸.

§Synthesised using HF-pyridine debenzylation method.

¶Synthesised using the HBr debenzylation method.

Table 1 summarises the data on the amino acid composition, molecular weight distribution and α -helical content of the synthetic polypeptide. Compared with the flounder's protein, the synthetic analogue is found to have a larger average molecular weight and a much lower α -helical content; the molecular weight of the protein is about 10,000 and its α -helical content about 85% (ref. 8).

Figure 1 shows the antifreeze activity of the synthetic polypeptide plotted as a function of its concentration in 0.01 M KCl, pH 6.0: the corresponding data for the flounder antifreeze protein are also shown. The synthetic polypeptide is apparently 'active', that is, like the antifreeze proteins, it depresses the freezing point of the solution to a far greater extent than can be accounted for in terms of its colligative property. This is illustrated by the data shown in Fig. 1 for serum albumin (molecular weight 68,000), which does not exhibit any antifreeze activity. Other globular proteins like myoglobin and cytochrome *c* were also inactive. An interesting feature of the data in Fig. 1 is the indication of a plateau in the activity of the synthetic polypeptide at higher concentrations, again very similar to what is observed with the protein counterparts (refs 1 to 4 and unpublished work of C. L. H., C. C. Yip & G. Fletcher). The magnitude of the antifreeze activity of the synthetic analogue is about one-third that of the flounder's protein at a given concentration. The decreased activity of the synthetic polypeptide when compared with the protein may be due to the

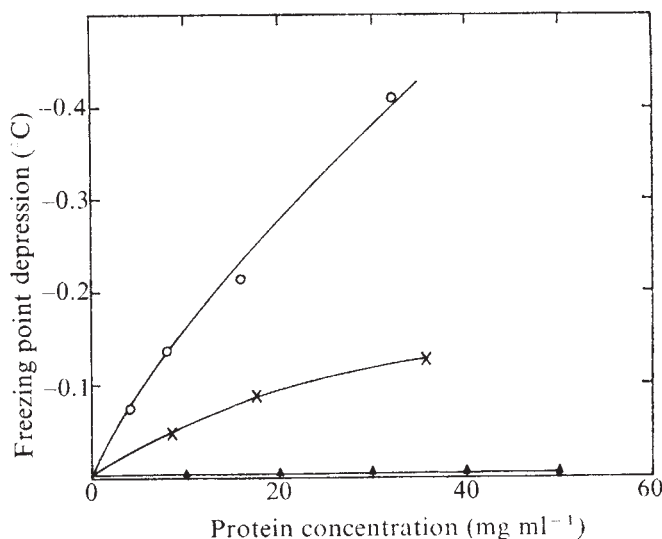


Fig. 1 Antifreeze activity of the synthetic random copolypeptide (x), SAF-I as a function of its concentration. The sample was dissolved in about 0.5–1.0 ml of 0.01 M KCl, pH 6.0, to yield the highest required concentration and dialysed extensively against the same solvent. The activity was determined as depression of the freezing point of the solution, using an Advanced Instruments model 3-R osmometer. Correction was made for the KCl in the solvent. The concentration of the polypeptide was determined by using $E^{1\%}_{1\text{cm}} = 18$ at 230 nm (ref. 8). The antifreeze activities of the flounder's protein (o) (unpublished work of C. L. H., C. C. Yip & G. Fletcher) and albumin (Δ) are shown for comparison.

differences in their amino acid compositions, sequences and molecular weights as well as the secondary structure. But the fact that such a simple model polypeptide can, albeit partially, mimic the natural protein in its function, seems to indicate the importance of the predominance of alanine in all the known antifreeze proteins. While we do not have an immediate answer as to the role of either the alanyl or aspartic residue in the mechanism of action of these proteins, a systematic study of the properties of the synthetic polypeptide analogues will, as pointed out by Feeney¹, prove to be valuable in our understanding of these proteins. Such a study is now in progress in our laboratories.

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Effect of light on artificial lipid membranes modified by photoreceptor membrane fragments

THE molecular basis of photoreception has been extensively studied, but it is not yet clear which of the photolysis products of rhodopsin has the key role in receptor potential generation. Penn and Hagins¹ have shown that the effect of an absorbed photon persists for about 0.1 s at 33 °C (rat retina). If metarhodopsin II is the key product, as most workers assume it to be, then the result of Penn and Hagins can be explained as follows. On formation of metarhodopsin II a visual pigment molecule undergoes a transformation with time constant 0.1 s, which 'cuts-off' the process of visual excitation. Metarhodopsin II persists for dozens of seconds at 33 °C (as determined from spectral characteristics²) so if the suggested transformation takes place it should not result in variations of the absorption spectrum of metarhodopsin II, and thus could not be detected by spectroscopy. As the artificial lipid membrane (ALM) is a good detector of changes in functioning protein molecules³⁻⁵, we attempted to detect the above process by examining the photoeffect on ALM, modified by photoreceptor membrane components.

Such an approach seems to be verified also by the fact that rhodopsin is a typical membrane protein, and investigation of the above model might give information useful for understanding the mechanisms of receptor potential generation. We used ultrasonicated rod outer segment fragments (ROSF) for the modification.

ROSFs were obtained by ultrasonic treatment of ROS suspension in distilled water. The membranes were formed using the method of Mueller *et al.*⁶ on a hole (1-mm diameter) of a Teflon cup placed in a glass chamber. The lipid solution used contained egg lecithin (18 mg ml⁻¹) and cholesterol (12 mg ml⁻¹) in heptane. The modification was carried out in the solution containing ROSF (absorbance 0.15 at 500 nm). The solutions on each side of the membrane were the same in all cases. A current through ALM was measured using an electrometer amplifier (Keithley model 301) in voltage-clamp conditions. The capacity of the modified ALM was two to three times lower than that of unmodified membranes (membrane capacity was evaluated from the time constant of the current recording circuit). The dark d.c. resistance of both modified and unmodified membranes was of the order of $5 \times 10^{10} \Omega$. 1-ms flashes from a xenon discharge tube were used to illuminate ALM.

The response of the modified membrane to a light flash is shown in Fig. 1 which shows that a flash brings about an increase of current through the membrane. A characteristic feature of the response is its reversibility—an increase of the current is followed by a spontaneous drop to the dark level. In the conditions of illumination used (bleaching of no less than 80% of rhodopsin), the effect could be observed only once for each modified membrane: repeatedly flashed white-light illumination caused no photoelectric current. Illumination by red light (beyond 650 nm) affected neither the current through the membrane, nor its ability to respond to a subsequent illumination by white light. In unmodified membranes, no photoeffect could be observed.

Further investigation of the photoeffect has shown the following:

- (1) The effect was independent of purity of the preparations. In the case of low-purity ROS (the purity criterion measured according to Heller⁷ being about 4.0), the membrane response was the same as with pure preparations (purity about 2.2). This indicates that a substance responsible for photosensitivity of ALM is contained in the outer segment.
- (2) The direction of current through the membrane was

determined by the applied voltage, and its magnitude always increased in response to light. At zero applied voltage, there was no current and no response. These facts show that the illumination of ALM brings about an increase in the membrane conductivity rather than the appearance of a photo-electromotive force, which had been observed for membranes containing carotenoids^{8,9}.

(3) Hydroxylamine at 100 mM did not change the observed photoeffect.

(4) A steady-state conductivity of a modified membrane was generally somewhat higher after illumination than before illumination.

(5) The photoeffect was observed, with no significant changes, in 100-mM NaCl and in 100-mM KCl (both solutions buffered with 10-mM imidazole/HCl, pH 6.25). The addition of Ca²⁺ to these solutions at 10 mM did not produce any detectable changes in the photo-induced conductivity. Addition of Ca²⁺ at higher concentrations resulted in a rapid aggregation of ROSF.

Investigation of the photoeffect in ALM at various temperatures gave the following results:

(1) The time constant of the photo-induced increase in the ALM conductivity was, within experimental error, the same as the period of formation of metarhodopsin II, cited in the literature (refs 10 and 11). An activation energy of the process was about 30 kcalorie mol⁻¹.

(2) The rate of the subsequent decrease in the model membrane conductivity depended on temperature according to the Arrhenius' law, with activation energy 19 ± 3 kcalorie mol⁻¹ (Fig. 2). From the extrapolation of the Arrhenius' plot to 33 °C, the time constant of the process was evaluated to be 80 ± 10 ms. Our data thus indicate that in the modified ALM light induces a process with time constant close to the duration of "a photon effect" as determined by Penn and Hagins¹. The observed return of the model membrane conductivity to a near dark level may therefore be con-

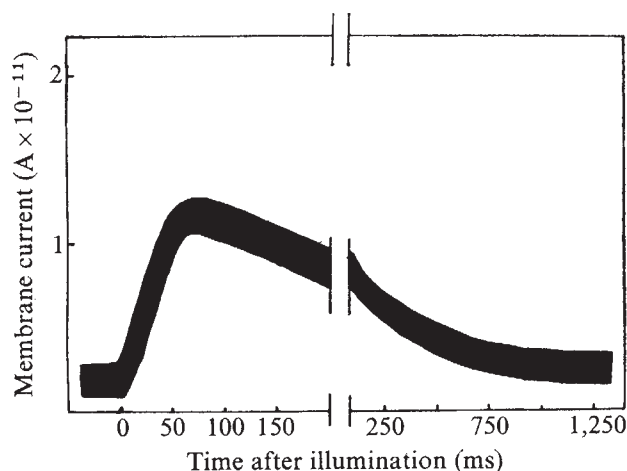


Fig. 1 The effect of illumination on artificial lipid membrane modified by ROSF. Conditions: 100 mM NaCl, 10 mM imidazole/HCl, pH 6.25; 17 °C. Voltage across the membrane, 100 mV. Recording time constant for the current flowing through the membrane—about 2 ms. ROS were isolated as follows: retinæ, excised from dark-adapted cattle eyes, were put in isoosmotic buffer solution (140 mM NaCl, 3.5 mM KCl, 1.8 mM CaCl₂, 0.5 mM MgCl₂, 10 mM Tris, pH 7.4) identical to that used by Korenbrot and Cone¹³, 1 ml per retina, and subjected to mild homogenisation on a magnetic stirrer for 20 min. The homogenate was centrifuged for 10 min at 100g. The supernatant was collected and centrifuged for 10 min at 10000g. The sediment was suspended in 40% (w/v) sucrose solution prepared in the isoosmotic buffer and subjected to flotation for 30 min at 30,000g. The flotation was repeated twice. To determine the optical density and the purity criterion, the ROS were solubilised with 2% solution of cetyl trimethylammonium bromide.

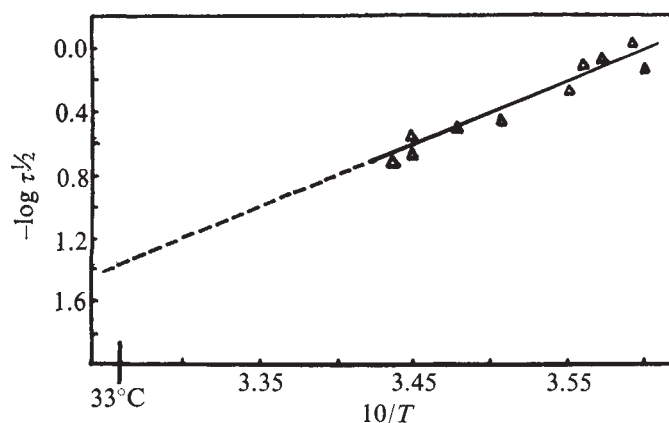


Fig. 2 Temperature (T) dependence of period (in seconds) required for the return of model membrane conductivity to half-maximum level ($\tau_{1/2}$).

nected with a 'switching off' of a mechanism inducing the receptor potential of a rod. No definite conclusions could be made as to the nature of the process involved, however. The mechanism probably involves either a conformation transition in rhodopsin after the formation of metarhodopsin II, or a rearrangement of photoreceptor membrane occurring, following a certain delay, after the formation of metarhodopsin II.

What is the mechanism of the observed photoeffect? The answer would depend on which situation was realised in our experiments—whether visual pigment molecules (or disk membrane fragments) are included in ALM, or ROSF in the shell form are simply sorbed at the model membrane. If the former is true, then our data apparently indicate that rhodopsin can modulate photoreceptor membrane permeability. In that case, one could suggest the simple realisation of a transmitter scheme of the photoreceptor function (for example, see ref. 12): a short-period photo-induced increase in the disk membrane permeability results in the release of a quantity of mediator capable of changing the permeability of ROS plasma membrane. If the latter version is the case, the variations in ALM conductivity most probably do not represent changes in disk membrane conductivity, and the model membrane would act only as a detector of rearrangements in visual pigment and (or) its lipid surroundings.

As yet it is impossible to make a choice between the two alternative mechanisms of photoeffect observed.

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Effect of aminoacylation on the conformation of yeast phenylalanine tRNA

MUCH effort has been devoted to analysing the effect of aminoacylation on the structure of transfer ribonucleic acid (tRNA). While it is generally held that changes in conformation provide the basis for the discrimination between acylated and non-acylated tRNA in tRNA: aminoacyl tRNA synthetase, tRNA: ribosome and tRNA: co-repressor interactions, the evidence to support this view to date is in serious conflict. We have been using the technique of laser light scattering to investigate the tertiary structure of tRNA (ref. 1). This technique is particularly well suited for analysing structural changes in macromolecules such as tRNA as it can detect changes of as little as 1% in the translational diffusion constant. The method has the advantage of allowing rapid, continual data monitoring making it possible to study dynamic changes in conformation. We describe here the use of this technique to monitor the diffusion coefficient of aminoacylated tRNA in conditions where deacylation occurs spontaneously. The results clearly demonstrate that: (1) large changes in the conformation of yeast phenylalanine tRNA do occur on aminoacylation, (2) the change can be reduced to an undetectable value by small changes in the solution parameters, and (3), the results apparently provide an explanation for many of the discrepancies observed by others.

Figure 1 shows the results obtained when phenylalanyl-tRNA was allowed to deacylate in the two different solution conditions. When the tRNA was in 10 mM Mg^{2+} , $D_{20,w}$ increased linearly from $7.46 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ at 555 pmol per A_{260} to $7.79 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ at 35 pmol per A_{260} . In this condition, the half-time for deacylation was 71 min. A least squares fit to these data yields an overall change in $D_{20,w}$ of $4.6 \pm 0.6\%$ with an extrapolated change of $14 \pm 2\%$ starting with pure, fully aminoacylated tRNA. On the other hand, when deacylation was allowed to occur in 1 mM $MgCl_2$ the values of $D_{20,w}$ showed no change as the extent of acylation decreased from 620 pmol per A_{260} to less than 1 pmol per A_{260} . In this condition the half-time of deacylation was 55 min. The slope of the plotted line was calculated to be zero within limits of error ($-0.5 \pm 0.9\%$).

The results show that the diffusion constant for yeast tRNA^{Phe} increases on deacylation in 10 mM Mg^{2+} . This change indicates that deacylation results in the formation of a more compact structure than that which obtains for the aminoacylated conformer. This interpretation agrees with results of Kaji and Tanaka² which indicated that aminoacylated *Escherichia coli* tRNA^{Phe} sediments slower than the uncharged species.

Of the interpretations consistent with our observations, the most plausible are that aminoacylation results in: (1) a disruption of secondary base pairing with resultant changes in secondary and tertiary structure or (2) changes in tertiary structure alone owing to a disruption of tertiary base pairing and/or an alteration in the distribution of counterions. The possibility that the observed changes in $D_{20,w}$ for tRNA^{Phe} in 10 mM Mg^{2+} is simply due to the addition of a molecule of phenylalanine can be ruled out due to the large magnitude of the change. The first interpretation also seems unlikely in light of results from a number of circular dichroism studies performed under a variety of solution conditions which indicate that no changes occur in the secondary structure on aminoacylation^{3–6}.

Alteration of the tertiary structure alone thus seems most plausible and while the results of this experiment do not allow us to specify the portion(s) of the tertiary structure involved in the change, results from other studies provide some insight. Two studies have shown increased steroid⁷ and polycytidine⁸ binding to the aminoacylated form of many tRNAs. Both ligands selectively bind guanosine residues suggesting that aminoacylation exposes the pair of guanosine residues which occur in the dihydrouridine loops of nearly all tRNAs⁹. In addition, results from studies on the binding of complementary

tetranucleotides to yeast tRNA^{Phe} strongly suggest that residues in the dihydrouridine and pseudouridine loops are exposed on aminoacylation¹⁰.

Other results indicate that changes can also occur in the region of the junction of the D-stem and amino acid acceptor stem. Differences were found in the circular dichroism band associated with 4-thiouracil when the spectra of charged and uncharged *E. coli* tRNA^{Phe} were compared¹¹. EPR studies with *E. coli* tRNA^{Phe} have shown a difference on aminoacylation of the rotational correlation times from a spin label attached to 4-thiouracil¹².

Taken together, these results suggest that aminoacylation

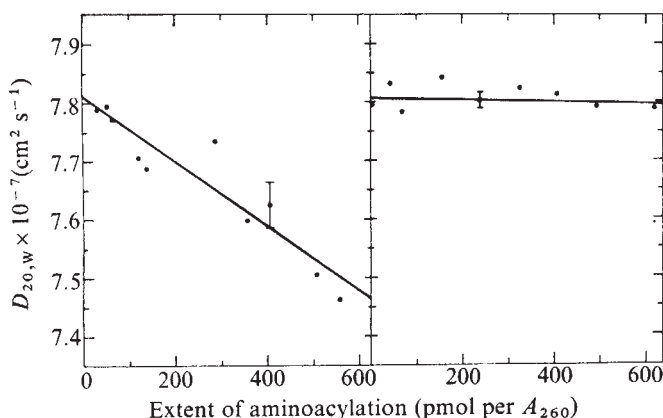


Fig. 1 Translation diffusion constant ($D_{20,w}$) of yeast tRNA^{Phe} as a function of the extent of aminoacylation. Phenylalanine tRNA with an acceptor activity of 850 pmol per absorbance unit (A_{260}) was aminoacylated with ³H-phenylalanine (200 mC mmol⁻¹) in a reaction mixture that contained, per ml: 25 μmol Tris-HCl, pH 7.2, 12.5 μmol MgCl₂, 4 μmol ATP, 5 nmol ³H-phenylalanine, 2 nmol tRNA^{Phe} and sufficient phenylalanyl-tRNA synthetase to allow complete charging in 15 min at 37 °C. When acylation was complete, 0.1 volume of 20% potassium acetate, pH 4.5, was added to the reaction mixture and the aminoacyl-tRNA repurified by chromatography on a small column of DEAE-cellulose. The tRNA was precipitated with ethanol, recovered by centrifugation, dried under vacuum and stored at -20 °C until use, usually within 18 h. In a typical experiment, 5 A_{260} (250 μg) of charged tRNA^{Phe} were solubilised in 25 μl of 1 mM sodium acetate, pH 4.5 and dialysed against the same buffer with 1.0 or 10 mM Mg²⁺. One-fifth volume of a 5 × stock of the appropriate buffer was then added: 8 μl of the sample were filtered into a 1 × 1 × 45-mm cuvette for light scattering; the remainder was filtered into a test tube thermostatically maintained at the same temperature (20 °C). Portions of the larger sample were withdrawn at various times, acidified with trichloroacetic acid and the acid-insoluble radioactivity collected on glass fibre filters to determine the extent of deacylation. Details of the procedures used in the purification of the tRNA and synthetase, the preparation of the ³H-phenylalanyl-tRNA and the preparation of the tRNA for light scattering will be described elsewhere. The techniques for data collection and analysis have been described¹. The concentration of tRNA for the experiment done with 10 mM Mg²⁺ was approximately twice that used for the measurement taken at 1 mM Mg²⁺. Hence, extrapolation of the diffusion constant values to zero tRNA concentration would yield a higher value with 1 mM Mg²⁺ than with 10 mM Mg²⁺. That extrapolation was not done here because an unknown, but constant amount of stray light was present due to the very small size of the cuvettes used for the measurements. The effect of stray light is to lower observed diffusion constant values by about 2% for every 1% of stray light present and is more significant at low concentrations of tRNA where the relative contribution by the stray light increases. These effects were not important in the large cuvettes used in previous experiments¹. Measurements were made at 20 °C for samples in 0.01 M Tris-HCl (pH 7.2), 0.1 M NaCl with 10 mM MgCl₂ and 6.2 mg ml⁻¹ tRNA (a) or 1 mM MgCl₂ and 3.0 mg ml⁻¹ tRNA (b). Each point represents an average of at least six determinations made within a 10-min period. Values of diffusion constant are normalised to 20 °C and the viscosity of pure water. Error bars represent ± 1 s.d. (σ) about the point; σ showed little variation during the experiment, with values consistently less than 0.3% of $D_{20,w}$.

may result in the disruption of tertiary base pairs between the dihydrouridine and pseudouridine loops and rearrangement of the 'L' structure to a less compact conformation. An apparent exception to this interpretation comes from results of a nuclear magnetic resonance study which showed no changes in hydrogen bonding of yeast tRNA^{Phe} on aminoacylation¹³. This study was performed at pH 5.0, however, a condition in which others could not detect a change in sedimentation behaviour on aminoacylation¹⁴.

Less likely, because of the magnitude of the effect, is the possibility that the observed changes occurring on aminoacylation are due primarily to an alteration in the distribution of counterions. Ninio *et al.*¹⁵ using X-ray scattering, concluded that intensity changes attendant with aminoacylation were probably due to perturbations in the layer of counterions—an effect eliminated at low Mg²⁺ concentrations. Consistent with this view, Cohn *et al.*¹⁶ found that the number of manganese binding sites changed with aminoacylation. Thus, it is possible that aminoacylation results in minor rearrangements of structure which alter divalent cation binding sites. This in turn could affect the distribution of counterions and hence, the diffusion constant. These interpretations are also consistent with the negative results obtained in a number of studies designed to detect changes in secondary structure^{3-6,13,17-19}.

The results presented here do not allow us to discriminate between changes in tertiary structure and/or counterion distribution. It is, however, possible to determine the contribution of each effect on the diffusion constant by performing this experiment at various concentrations of tRNA and salt. These experiments are in progress. The present results vividly demonstrate the importance of Mg²⁺ ions in structural changes of tRNA^{Phe} on aminoacylation. Magnesium ions have been implicated previously in structural changes of deacylated tRNA^{Phe} (ref. 1); however, this is the first clear evidence that the conformational changes which occur on aminoacylation/deacylation are altered by magnesium. The results also suggest that specific changes in intracellular magnesium levels might have a central role in regulation of tRNA function.

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matters arising

The Star of Bethlehem

I WOULD like to make a brief comment on David Hughes's article on the Star of Bethlehem. While the astronomical phenomena are described with vivid precision, the historical aspect would merit equal attention. Dionysius Exiguus is surely thought to have placed the Annunciation at 25 March 1 BC and the Nativity therefore at 25 December 1 BC and not AD 1. I quote from E. J. Bickerman¹ in support of my view that Dionysius' supposed 5-yr error is at best an irrelevance (page 77 of ref. 1).

An era *ab urbe condita* (from the founding of the city of Rome) did not in reality exist in the ancient world, and the use of reckoning the years in this way is modern. . . . The principal reason for not using the system *ab urbe condita* was that the age of the city was disputed: *est enim inter scriptores de numero annorum controversia* (Cic. *Brut.* 18, 72). The date of the founding in Roman historiography—excluding the more extreme opinions—oscillates between 759 and 748 BC.

Of 58 modern authors cited by Holzmeister², 33 select for the year of the Nativity 7–5 BC, so Hughes represents majority modern opinion. Of 42 ancient opinions listed, however, not one falls as early as this period and only one before the supposed date of Herod's death. A thorough re-examination of the date of the Nativity must also ask whether it is certain that Herod died in 4 BC. Josephus states that Herod reigned 34 years from the death of his predecessor, executed by Antony some time after the capture of Jerusalem which fell in 37 BC. The obvious arithmetical error is accounted for by the explanation that Josephus counted the year in which a king died and the (same) year of the accession of his successor as two separate years, and therefore, overestimated the length of each reign by 1 year—his so-called 'inclusive' method.

Although this notion of Josephus' mode of computation is the pillar on which the modern opinion rests, no shred of evidence is adduced for it, for there is none. Evidence to the contrary is, however, easily found in *Jewish Antiquities*. Between February 135 BC and October 63 BC there were in Jerusalem six rulers in the 72 years 8 months. Summing the lengths of reigns as given by Josephus, we have 71 years 6 months. This is very exact and certainly evidence that Josephus did not reckon 'inclusively'.

Since Herod died shortly after a lunar

eclipse, the times are restricted in which his death could have fallen. 4 BC is too early to allow for a reign of 34 years (if the 'inclusive' method of counting is rejected) and there were no such eclipses in 3 or 2 BC. It is then hard to escape from the conclusion that Herod died following a partial eclipse in 9/10 January 1 BC having completed 34 years' reign a few months earlier. Just before the eclipse, there was an uproar in the Temple recorded by Josephus, and possibly unleashed by the advent of the Magi if the traditional date of the Adoration, 6 January is again considered.

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¹ Bickerman, E. J. *Chronology of the Ancient World* (Thames and Hudson, London, 1968).

² Holzmeister, U. *Chronologia Vitae Christi* (Rome, 1933).

HUGHES's suggestion that the 'Star of Bethlehem' was probably a triple conjunction of Saturn and Jupiter in the constellation Pisces in 7 BC was anticipated by Stauffer in 1957 (*Jesus: Gestalt und Geschichte*). The significance of this '*coniunctio magna*' was explained as follows: Jupiter was the 'star' of the world ruler and the constellation of the fishes as the sign of the last days; the planet Saturn was considered in the east to be the 'star' of Palestine. So the conjunction indicated, not a 'Jewish king born in Israel', but that in that year there would appear in Palestine the ruler of the world in the last days.

As long ago as 1835 D. F. Strauss observed the evangelist's contrived story to show fulfilment of the prophecy of Micah that the ruler of Israel should come from Bethlehem, and claimed "we have absolutely no guarantee that Bethlehem was his birth-place" (*The Life of Jesus Critically Examined*, SCM ed. 1973, p 156). Guignebert concluded that the magi, star, massacre of innocents, birth in a stable, shepherds, angels, and so on, are all unhistorical and that we do not know where Jesus was born, except in Galilee. He also noted that the idea that the Jewish population had to move all over the land to register for the taxation was "outside the plane of reality" (*Jesus* 1935, pp. 94 & 99). Mackinnon noted the 'assumption' that enrolment required the parents of Jesus to journey to Bethlehem for this purpose, and did not believe the stories of the magi and

the massacre of infants (*The Historic Jesus*, 1931, pp. 19 & 24). More recently, Gunther Bornkamm, in his *Jesus of Nazareth* (1951) says hardly anything about the birth, infancy or early years of Jesus. He claims that the birth and infancy stories of Matthew and Luke are largely legendary in character and do not contribute to a *History of Jesus*.

All that we know about Jesus is that he was born in Galilee. If he was about 30 years old (Luke III, 23) when John baptised him in Tiberius's 15th year (Luke III, 1) then it looks as if he was born in the terminal year, and that the chronology of Dionysius Exiguus is correct. We know of no reason why this should not have been so, and there is no cause to believe the stories associating the birth with celestial signs or the Roman registration of 6 CE.

Incidentally, Dionysius did not "forget the year zero"; there was no such year, as demonstrated by Hughes's own chart!

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KEPLER may not have been the first to be impressed by a Jupiter-Saturn appulse¹. In the chronicles of Worcester Priory (1377) a conjunction in Aquarius was recorded in 1285—"it had not happened since the Incarnation". Newton² comments that this suggests that the Star "had already been interpreted as a conjunction by AD 1285". It could be a rhetorical expression.

The object of -5 (6 BC) is regarded by Stephenson³ as a comet, because there is a possible reference to a tail and motion and certainly there does not seem to be any remnant known.

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¹ Hughes, D. W. *Nature* 264, 513–517 (1976).

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I COMMENT here on two points made by David Hughes. First, there seems to be no need for a year zero because from our standpoint, in 1976, any year in which Jesus Christ was born, be it spring or autumn, was 1 AD—"the first year of the Lord," and the preceding year 1 BC—"the first year before Christ." Perhaps the author thinks of

1 AD as the first year after the birth of Christ.

Second, for those of us who hate to believe that St Luke or any Holy Scripture could have been "mistaken", or at least so easily written off as such, perhaps Cyrenius and Quirinius are not one and the same.

With no year zero to account for between 1 BC and AD 1, the author's second scenario seems more likely to conform to fact.

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HUGHES, in his article on the Star of Bethlehem, makes life unnecessarily difficult for himself. He estimates that a journey of 550 miles across the desert, from Sepharuaim to Jerusalem would have taken four months in preparation and travelling. Consequently he has to show that the triple conjunction of Jupiter and Saturn in Pisces would have been visible, in varying positions, throughout that period of time.

T. E. Lawrence, writing of the Arab Revolt in *Seven Pillars of Wisdom*, recorded that a fully loaded camel, under an experienced rider, could, if hard pressed, cover 80 to 100 miles in a 24-hour period, depending on the country, and that 50 miles ridden in the same period was considered a holiday by comparison. Even the most inexperienced and clumsy riders, who positively hindered their animals, could travel 30 miles a day. The length of stages undertaken in the crossing of a desert is limited, of course, by the distance between wells, but even so, the Magi could have completed their journey from Babylonia in 2 weeks at the outside, or in 10 days with less comfort. Allowing 2 weeks for preparation (gathering of stores, hiring of men and beasts and so on), the whole enterprise would have taken a month at the most.

In other words, the Magi not only could have set off when the triple conjunction was at its best, but would also have been in a position to respond to signs of a much more transient nature.

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HUGHES' convincingly dates Jesus' birth by astronomy to 7 BC but unfortunately rejects the historical evidence in Luke II, 2, as usually found in English versions, when an equally valid and grammatically justified alternative translation can be used to support his theory. Luke II, 2 is usually translated "This was the first enrolment, when

Quirinius was governor of Syria". An enrolment, or census, under Quirinius is well documented because of a subsequent rebellion³⁻⁵, but Quirinius was not governor until AD 6-9. Thus, Luke seems to contradict both the astronomy and Matthew (II, 1), who says Jesus was born before Herod the Great died, that is, before 4 BC.

The necessary change, which completely alters Luke's evidence depends on the use of the Greek word *protos*, usually translated 'first'. *Protos* is the superlative derived from *pro*, meaning before, the comparative being *proteros*, meaning former or sooner. Consequently, according to strict grammar, *protos* should mean first of at least three. But the Hellenistic Greek used in New Testament times was at least as relaxed as modern English in which 'first' is often used when 'former' or 'prior' would be more grammatical⁶. Thus, 'first enrolment' in its Hellenistic connotation is 'first of two' or 'one before' and, as Bruce⁷ suggests, Luke II, 2 is best translated "This enrolment was before that made when Quirinius was governor of Syria". Similar expansions, in which words need to be supplied in translation, are not uncommon and there is no grammatical reason against supplying them in this passage⁸.

Numerous Egyptian papyri of a slightly later date indicate that enrolments occurred every 14 years (ref. 3). If this 14-year interval was observed in Syria the enrolment before that of Quirinius would have been sometime between 9 and 6 BC. Thus, Luke II, 2, instead of being dismissed as a mistake, can be used to support the date deduced by Hughes.

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¹ Hughes, D. W. *Nature* 264, 513-517 (1976).

² Bible, Revised Standard Version (quoted as a modern literal translation).

³ Souter, A. & Baker, J. C. in J. Hastings *Dictionary of the Bible* 2nd edn (Clark, Edinburgh, 1963).

⁴ Bruce, F. F. *New Testament History* 90-91 (Nelson, London, 1969).

⁵ Acts, V, 37, also written by Luke.

⁶ Turner, N. *Grammatical Insights into the New Testament* 23-24 (Clark, Edinburgh, 1963).

⁷ Bruce, F. F. *New Testament History* 30 (Nelson, London, 1969).

HUGHES REPLIES—The significance of the triple conjunction of Jupiter and Saturn in the constellation of Pisces' around 7 BC can be better judged by reference to Fig. 1. At no time do the planets approach closer than 1°, about twice the diameter of the full moon. All the stars in this region pale into significance in comparison with the brightness of the two planets (see Fig. 2).

'Herod' was a fairly common name in those times. Herod the Great, the son of Antipater, was made the Governor of Galilee and afterwards titular King of Palestine by the Romans. On his

death the kingdom was divided between his three sons, Archelaus, Herod Antipas (who received Galilee) and Herod Philip II. There is very little doubt that Jesus was born during the reign of Herod the Great (Matthew II, 22) and was tried and crucified during the reign of Herod Antipas (Luke XXIII, 8, Matthew XIV, 1); however, some biblical chronologists do cast doubt on the generally accepted superstition that Herod the Great died between 13 March and 11 April 4 BC, as Edwards shows above.

Simmons finds the month of the birth of Christ by relating the lives of Christ and John the Baptist (personal communication). Zacharias, the father of John the Baptist, was a priest of the course of Abia (Luke I, 8) and would have served in the temple during the 6th week after Passover, the week before Pentecost. As all the priests also served during Pentecost, Zacharias would have left Jerusalem for his home town around Sivan 12th (15 June). Elizabeth, his wife, conceived soon after his return (Luke I, 24) so John the Baptist would have been born about 280 days later, around 27 March. Luke (I, 36) records that Christ was 6 months younger than John the Baptist so this puts the birth of Christ in late September. Interestingly we read in the Talmud that sheep were taken in from the hills on about the first of November until March so shepherds would not be on the hills watching over their sheep between these dates.

Addey⁹, using the same evidence as Hughes' comes to a similar conclusion, that Christ was born in the Autumn of 7 BC. Using traditional astrology (apparently giving Christ's horoscope a strong Sun/Leo/fifth house element) and the early Christian tradition that Jesus was born on the day after the Jewish Sabbath, that is on a Sunday, Addey finds the characteristics of Jesus are best fitted by the horoscope for the evening of Saturday 22 August 7 BC (note that in the Jewish calendar the

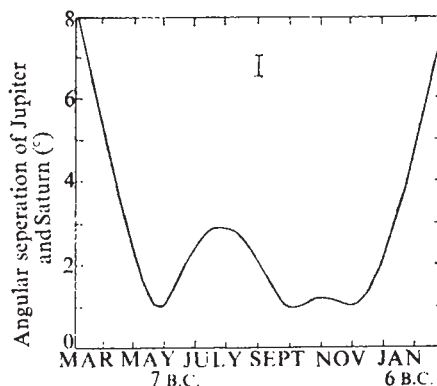


Fig. 1 The angular distance between Saturn and Jupiter during the triple conjunction of 7 BC. The scale bar represents the diameter of the moon in the same scale.

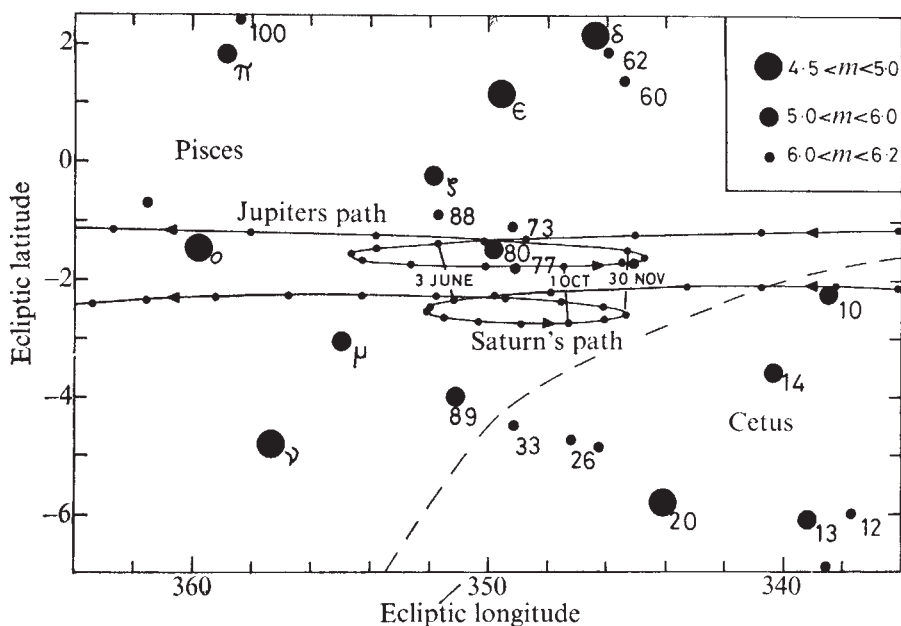


Fig. 2 The 7 BC triple conjunction of Saturn and Jupiter in Pisces. The ecliptic latitude and longitude have been taken from Tuckerman². The dots on the planetary paths are separated by 20-day intervals; m is the apparent stellar magnitude.

day changes at sunset). The legend that Christ was born shortly after sunset, which originates from the Arabic Gospel of the Infancy, is also mentioned by Kestranek⁶. The mystical writings in *The Urantia Book*⁷ give the time of Christ's birth as noon on 21 August 7 BC.

A novel hypothesis that has also been considered is that there were two Messiahs, both named Jesus born at slightly different times (ref. 8). Edwards supports this, pointing out that the nativity in Luke belongs as firmly to the reign of Archelaus as the Matthean nativity does to the reign of Herod the Great (O. Edwards, personal communication). Steiner⁸ maintains that Matthew describes the birth of one Jesus, who was visited by the Magi whilst Luke describes the birth of a second Jesus who was born a little later and was visited by the shepherds and eventually crucified. The evidence he brings forward to support this claim is that Matthew and Luke give different genealogies, in Matthew Jesus being descendant from Solomon whilst in Luke He is traced back to Nathan. Second, in Matthew Jesus is born in a house and is visited by wise men, in Luke He is born in a stable and visited by shepherds. Third, Matthew describes the slaughter of the innocents and the flight into Egypt whilst this is not mentioned at all in Luke. This plurality of Messiahs was apparently concealed by Jerome and the newly official Church of the Roman Empire when they juggled with the different genealogies and expurgated the Infancy Gospels to produce a simple creed for the masses on whom the Church of

the Roman Empire had been newly imposed. The expectation of the coming of two Messiah was often mentioned in the Dead Sea Scrolls found at Qumran in 1947.

Montefiore⁹ considers another important point from ancient Jewish tradition. The Messiah-Haggadah¹⁰ discusses the heptad in which the Son of David will come and states that in the fifth year "the Star shall shine forth from the East and this is the Star of the Messiah . . . and at the close of the seventh the Messiah is to be expected". This and other statements in the Midrashim in Jellinek's collection¹⁰ indicate that a Star in the East was to appear two years before the birth of the Messiah. This ties in rather interestingly with Matthew II, 16 "Then Herod, when he saw that he was mocked of the wise men, was exceeding wrath and sent forth, and slew all the children that were in Bethlehem, and in all the coasts thereof, from 2 years old and under, according to the time which he had diligently enquired of the wise men". This "time" was obviously not the time of Christ's birth, which the Magi did not know when they first visited Herod, but was the time of the first appearance of the star. Using the triple conjunction hypothesis this occurred on 29 May 7 BC. Taking the old tradition that this would occur 2 years before the birth of the Messiah, Herod concluded that Jesus could have been born at any time between 29 May 7 BC and 29 May 5 BC and killed all the children of 2 years and under to be on the safe side.

Palmer¹¹ is perfectly correct in stating that the Magi could (like Lawrence

of Arabia) have travelled from Babylon to Jerusalem in 10 to 14 days and this must be taken as the lower limit for the journey time. The four months' journey time was mentioned in ref. 1 because this was the time taken by the Israelites when they were lead across the desert by Jehova on their return from captivity in Babylon. There is also a strong possibility that the Magi did not travel directly across the desert but took the easier but more distant route around the fertile crescent (Babylon, Mari, Carchemish, Ugarit, Byblos, Jerusalem—see Keller¹²).

Finally, a philological point¹³. Matthew uses the word star (αστηρ) four times (Matthew II, 2, 7, 9, 10) and each time it is used in the singular. Now *αστηρ* is not a word that is spelt the same way in both its singular and plural forms (the plural is *αστερες*), nor is it a collective noun (*αστρον* being a constellation), so it should not have been used to refer to more than one star. Second, if the 'star' of Bethlehem was a conjunction of two planets why didn't Matthew refer to 'planets' (*πλανης αστηρ*, a wandering star) and not stars? In answering these two points it must be remembered that Matthew's native language was not Greek, and also that Greek of the New Testament is not the classical Greek but the *κοινη* or popular everyday Greek, so words might not have been used in their strictly classical sense. Also Matthew, like other New Testament authors adhered in style and usage to the language of the Old Testament. 'Star' appears 24 times in the Old Testament, the word planet not appearing at all. So Matthew might just have used the better known expression.

The Matthean use of the singular was thought by Gerhardt¹⁴ to refer simply to one planet in 7 BC, Saturn, the planet that was in conjunction with Jupiter. Saturn was "the protector of the Jews and reigned over their religion"—it was thus their star and the symbol of their Messiah.

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Sodium and chloride in rainwater

PATERSON and Scorer¹ have suggested that methods used in rainwater analysis for Na⁺ and Cl⁻ may give incorrect results and the increase in Na/Cl ratio reported by Hutton and Leslie² for inland samples collected in Victoria (Australia) relative to coastal samples may be an artefact. These suggestions are not acceptable.

Contamination of rainwater samples by Na is always possible and must be prevented as far as is practicable. In some Victorian samples Na was recorded at 0.2 µmol per 10⁻⁴ m³ with the limit of detection being 0.1 and thus Na contamination should not be significant in samples reported as having 2 µmol per 10⁻⁴ m³ or more. The limit of detection of Cl was 0.5 µmol per 10⁻⁴ m³ and this limit imposes an artificial restriction on the Na/Cl ratio in dilute samples. Seawater with a Na/Cl ionic ratio of 0.858 when diluted to a Cl concentration of 3 µmol per 10⁻⁴ m³ will have a ratio of 1 due to rounding to the nearest whole number and the value of *R_s* of 1.8 expected by Paterson and Scorer¹ will not be observed in at least 12 of the Victorian samples in their Fig. 3, it will be 1.5.

Nevertheless, there is enough evidence in the Victorian data to conclude that terrestrial sources contribute significant Na particularly at inland stations in summer and autumn. Hutton and Leslie² in their Fig. 3 show seasonal variations at six stations from coast to 320 km inland and in 14 out of the 17 sets of data the Na/Cl ratio in autumn is equal to or greater than the ratio in winter and in 9 out of the 11 sets of data the ratio in spring is equal to or less than the ratio in summer. Furthermore, the data in Table 1 give the following regression for 1955

$$\text{Na/Cl} = 0.89 + 0.0022 \times d$$

(*r*² = 0.529, *n* = 24) and for 1956

$$\text{Na/Cl} = 0.86 + 0.0008 \times d$$

(*r*² = 0.454, *n* = 8). (*d* = distance (km) from ocean in south-west direction). In both years at zero distance the value of the Na/Cl ratio is close to 0.86, the seawater ratio, and that in 1956, the wetter year for places usually receiving less than 625 mm of rain, the ratio changes less with distance.

It may be that the Eskdalemuir and Lerwick data (Fig. 2 in ref. 1) are not in error and the Wraymires site may be influenced by abnormal local factors.

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¹ Paterson, M. P. & Scorer, R. S. *Nature* 254, 491–495 (1975).

² Hutton, J. T. & Leslie, T. I. *Aust. J. Agric. Res.* 9, 492–507 (1958).

PATERSON REPLIES—Hutton has missed the point of our discussion¹ of the published data on ionic ratios of sodium and chloride in precipitation. Our analysis of data from a number of studies, most of them connected with the European Air Chemistry Network, found that current theories on segregation of these elements after originating in sea salt aerosol, were based on uncorroborated data. When independent results allowed consistency to be checked, no support was found for any real deviation from the seawater ratio of Cl/Na which is 1.80 by weight. If chlorine in some form is driven off the sea salt through chemical or photochemical action, it seems to be brought back again in any cloud droplets which form on the aerosol, as would be expected from the high solubility of the Cl₂ or HCl which are the postulated gaseous forms. The Australian data of Hutton and Leslie² was introduced into the discussion because their finding of a deviation from the sea salt ratio, which may be an important result as it has been found in the rather different environment of the dry and clear Australian atmosphere, also requires corroboration by an independent system of sample collection and analysis. Hutton's continuing failure to come to grips with this consideration is indicated by his final statement, made without supporting argument, that "the Eskdalemuir and Lerwick data (in ref. 1) (may not be) in error and the Wraymires site may be influenced by abnormal local factors."

Hutton's claim (refs 2, 3) that much of the sodium and chloride in inland samples arises from terrestrial sources is supported by a considerable weight of evidence, including the reanalysis of Hutton's Mildura data⁴ by Paterson⁵ which indicates that both elements are collected by that rain gauge at a rate which is effectively independent of the amount of rain during the month. This suggests that much of it has arrived as a dry deposit of aerosol, in quantities substantially greater than those found at a similar distance inland in Europe and North America. The range of atmospheric transport of these particles remains an open question, especially in view of Bigg's observation (personal communication) that extensive sampling from aircraft over wide areas of inland Australia has revealed very low concentrations of particles from any natural source. Perhaps the explanation is to be

found in Dulhunty's observation⁶ of the intermittent but intense dry storms which raise material from the dry salt lakes in brief episodes. Galbally (personal communication) has reported sightings from the air of 'salt devils' raised by willy willies crossing the dry lake beds.

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¹ Paterson, M. P. & Scorer, R. S. *Nature* 254, 491 (1975).

² Hutton, J. T. & Leslie, T. I. *Aust. J. Agric. Res.* 9, 492 (1958).

³ Hutton, J. T. *Nature*.

⁴ Hutton, J. T. *Arid Zone Research* 11, 285 (UNESCO, Paris, 1958).

⁵ Paterson, M. P. thesis, London Univ. (1975).

⁶ Dulhunty, R. *The Spell of Lake Eyre*, 5 (Lowden Publishing, Kilmore, 1975).

Habitat values and endemism in the vanishing rain forests of Sri Lanka

In our paper¹ the following scientific names were misspelled due to proof reading by committee. The correct spellings are *Nannophrys guentheri*, *Ramanella obscura*, *Cophotis ceylanica*, *Lyriocephalus scutatus*, *Otocryptis wiegmanni*, *Geckoella triedrus*, *S. taprobanensis*, *S. diegnani*, *Nessia burtoni* and *A. hickanala*.

Table 2 in the same paper was based on inaccurate estimates of the areas of the Regions. Our figures were obtained from the Department of Agriculture of Sri Lanka and were too large by a factor of about 1.4. We have prepared a more accurate rendition of the table using the areas in Kahawita² (Table 1), but since the ratios of the areas of the four regions are hardly affected the ratios of the habitat values and our conclusions are conserved.

We thank Dr H. Crusz at the Department of Zoology, University of Sri Lanka, Peradeniya, Sri Lanka, for pointing out the errors.

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¹ Senanayake, F. R., Soulé, M. & Senner, J. W. *Nature* 265, 351–354 (1977).

² Kahawita, R. *Proc. Ceyl. Ass. Adv. Sci.* 8th Annual Session, II, 63–85 (1953).

Table 1 Habitat value data and indices for the amphibians, lizards and birds of Sri Lanka

Region (km ²)	Area	Endemic Amphibians			Endemic lizards			Endemic birds			Totals		
		No.	<i>V_i</i>	<i>V'_i</i>	No.	<i>V_i</i>	<i>V'_i</i>	No.	<i>V_i</i>	<i>V'_i</i>	No.	<i>V_i</i>	<i>V'_i</i>
A	43,302	0	0	0	5	0.12	0.12	3	0.07	0.05	8	0.19	0.17
B	10,742	5	0.47	0.39	7	0.65	0.65	11	1.02	0.43	22	2.14	1.47
C	7,851	7	0.89	0.71	10	1.27	1.27	18	2.29	1.09	35	4.45	3.07
D	2,873	8	2.78	2.27	9	3.13	3.13	18	6.27	2.34	35	12.18	7.74

reviews

Physical space and time

D. J. Raine

Space and Time in the Modern Universe. By P. C. W. Davies. Pp. viii+232. (Cambridge University: Cambridge, London and New York, 1977.) Hardback £6.50; paperback £2.95.

THERE was a time when the sins of the world would be explained through Quantum Theory and excused by Relativity. But the relevance of science to society is not so crude, and in due course Heisenberg and Einstein and their friends were returned to Academe. Nor is the relevance of science to be measured solely in terms of the subsequent military and technological applications, for the General Theory of Relativity at least—in some contrast to the Special Theory—has as yet no such applicability. This much it shares with the Copernican theory. That new vision has not changed one bit the motion of the Sun, or helped us to burn its light. Yet, as the contemporary Church understood well that it would, in shaping the way we see ourselves in relation to the World, the Copernican view has influenced our lives more than any display of military genius or noble act of statesmanship. And General Relativity, as part of our newly forming picture of the space time of the Universe may yet prove to have a similar relevance.

In *Space and Time in the Modern Universe*, Dr Davies is concerned to communicate, in a basically non-mathematical form, the status of our present understanding of physical space and time. He takes the reader from the mathematical idea of the continuum through Newtonian mechanics to electromagnetic theory and the space time of Special Relativity; from Newtonian gravity to the space time of General Relativity; from the microscopic world of the quantum to the Universe as a whole. Among the unresolved problems to which we are introduced are the reconciliation of the time symmetry of physical laws and the time asymmetry of natural processes; the reconciliation of Quantum Theory and General Relativity, still far from understood despite the progress which has been made in the

quantum mechanics of black holes; and the reconciliation of the successes of General Relativity with the fact that it seems, in the singularity theorems, to predict its own inadequacy.

To encompass this vast range, Dr Davies adopts what one might call the synoptic approach. The danger of this style is the ease with which it becomes a mere recital of the table of contents of the book of physical thought. So wide-ranging is the discussion that the author's skill in making a point with a deftly chosen analogy or a succinct diagram does not always enable him to escape this pitfall. Thus, quasars are no more than "strange objects", spontaneous emission is not stimulated as in lasers, and escape velocity is "escape velocity".

In selecting concepts for further discussion—and clearly considerable selection is required—there is a wide variation in the demands made on the reader. For example, he is expected to understand that higher frequency photons have higher energies (mentioned in passing in a discussion of radiation pressure); or consider "... the presence of electrically conducting materials will modify the fluctuations of photons ('particles' of electromagnetic radiation)" which, albeit for the second time, contains the only definition of photons given and for which more than a passing acquaintance with physics is required. On the other hand, the book starts by distinguishing between space and "outer space"; great care is taken to explain that the Solar System consists of the Sun and nine planets; and can anyone who does not already know what an element is, be expected to find it clear that a hot gas emits X rays?

These might be thought to be minor blemishes from which not even a masterpiece would be entirely free. I feel, however, that they are more likely to be symptomatic of an underlying unresolved pedagogic problem. For example, if it is emphasised that Special Relativity is a macroscopic theory which does not apply in the atomic domain, how is anyone without a high degree of physical maturity to understand that it is by incorporating this

same relativity into the atomic description that Dirac discovered anti-matter? And compare the beautiful construction and dramatic effect of the chapter on time asymmetry with the visible discomfort at the need to explain the 'extraordinary' Quantum Theory *ab initio* in a few pages—and the almost audible sigh of relief on returning to relativity.

To be fair, it is indeed clearly stated that the book is addressed to those who already know enough of the subject to have asked themselves what might happen before the 'big bang' or at the centre of a black hole. Yet in the writing of the book, it seems that at times Dr Davies must have felt he might reach a wider audience. Certainly the results are of such importance that one can understand the desire to communicate them beyond the undergraduate physicist. But the problems are so difficult that it is probably impossible to satisfy entirely the whole of this wider audience in a single book.

In the final chapter of the book, Dr Davies turns to Man and the Universe. Here what matters is not so much the details of what has gone before but rather that it provides a reasonably coherent view of a Universe evolving. If the Universe is infinite and if the probability of the emergence of life is greater than zero, then there are infinitely many beginnings to Civilisation. It may be the destiny of some of these to evolve, to understand and perhaps to control their World. We are too young to know. If only a physicist would claim that the physical basis of life is now understood—and if Dr Davies is too optimistic in claiming that we already understand the evolution of structure in the Universe—nevertheless, such an understanding certainly seems to be emerging. And if we look outwards from ourselves to view this new Universe, this puts a unique complexion on our lives. What is important is that as many people as possible should be brought to know this view. □

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Intermediate astronomy

Astronomy: Principles and Practice. Pp. 341. *Astronomy: Structure of the Universe.* Pp. 283. By A. E. Roy and D. Clarke. (Adam Hilger: Bristol, UK, 1977.) Hardback £10; paperback £7.50 each volume.

ASTRONOMY owes its educational value to a combination of separate factors: historical and literary associations, a wealth of comparatively easily understood applications of the scientific method, an increasingly significant relationship with the environment as revealed by modern space research, and its fundamental role in exploring what sort of Universe we live in. As Plato said, astronomy is good for the soul, and increasing recognition of this fact has led in recent years to the production of a large number of lively and beautifully illustrated books directed at 'liberal arts' students and bright youngsters, often making little or no use of formal mathematics; a few of these have been reviewed in *Nature* (266, 102, 3 March, 1977) by D. W. Hughes. For those with the mathematical equipment of a first-year honours student in physical sciences, an admirable compact survey of the whole field is provided by A. Unsöld's *New Cosmos* (of which an English version of the second edition is currently in press), whereas numerous other books deal with particular aspects in more detail.

The present two volumes by Roy and Clarke aim at an intermediate level. They are based on university and extramural lectures given by the authors at Glasgow over a number of years, and contain essentially the material of the first-year university course attended by beginning science students (who study a range of subjects at this stage) and a minority of arts students wishing to gain some knowledge of the workings of science and the scientific method. The first volume, beginning with naked-eye phenomena and a brief description of the observables, proceeds in classic Glaswegian tradition to give a thorough grounding in elementary spherical trigonometry, positional astronomy and celestial mechanics, including useful topical references to Megalithic observatories and space-probe orbits. A short introduction to atomic and radiation physics leads on to the second major section discussing optics, optical and radio telescopes, detectors and instruments, with a final chapter describing a number of practical projects, mostly concerned with positional astronomy but with some optics and spectroscopy as well, that involve very simple equipment. The second volume describes the

objects and phenomena of astronomical research: members of the Solar System, tides and eclipses, stellar magnitudes, colours and spectroscopy, variable and binary stars, stellar atmospheres, interiors and evolution, stellar motions, nebulae, galactic structure, galaxies, cosmology, cosmogony and the question of extraterrestrial life.

Admirably illustrated and neatly packaged into 40 separate chapters with exercises, the two volumes are very successful in giving a clear exposition and in driving points home. The treatment and content, however, are not very well adapted to the needs of either honours students or liberal arts students, nor to those of bright youngsters. The mathematical approach is distinctly old-fashioned, with spherical triangles solved in remorseless detail (including logarithms of cosines written out in full), celestial mechanics without benefit of vector analysis, and trivial relationships involving stellar magnitudes spelled out at length.

The scope of the second volume is severely limited: to take just two ex-

amples, there is no mention of curves of growth or opacity in stellar atmospheres, and in the section on cosmology, General Relativity is merely mentioned as satisfying the classical observational tests without any account of the Principle of Equivalence or of any other idea connected with it, so that the notion of the curvature of space then comes right out of the blue. Thus the present book gives considerably less information on astrophysical topics than does the largely non-mathematical treatment of the entire field by G. O. Abell in *Exploration of the Universe*. The practical outlook, and the clarity and thoroughness of treatment (up to the chosen level) of those topics that have been selected for coverage, will, however, make this book a useful work of reference, especially for ambitious amateurs and for teachers of extramural courses.

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Viruses and cell surface changes

Cell Surface Reviews. Volume 2: Virus Infection and the Cell Surface. Edited by G. Poste and G. L. Nicolson. Pp. 342. (North-Holland: Amsterdam, New York and Oxford, 1977.) \$55.95; Dfl.137.

THE nature of surface changes induced by interactions between viruses and cell membranes is currently a topic of considerable interest to many virologists and cellular immunologists. This follows the realisation that cell-mediated immunity in virus diseases reflects surface monitoring of virally modified cells by re-circulating thymus-derived lymphocytes (T cells). Both viral and host components are apparently involved in T cell recognition. The evidence that has led to this concept is reviewed here by Blanden, Pang and Dunlop, and Burns and Allison. The area is, however, moving quickly and this volume is rather tardy, so some of the ideas expressed now have little currency.

The subject has not always been so fashionable. In fact, most of the virologists writing for this book are either unaware of, or have chosen to ignore, the trendy speculations of the cellular immunologists. This is somewhat of a relief. The result is a series of detailed reviews of established knowledge in areas that seem, in the main, to be relatively stable. The topics covered range through interactions of viruses with tissues (Smith, Weiner and John-

son), nucleated cells (Hosaka and Shimizu, Burns and Allison), erythrocytes (Bächi, Deas, and Howe) and model membrane systems (Tiffany). A thorough account of the structure and assembly of viral envelopes is provided by Rott and Klenk. Those considering the problem of immunological recognition of virally modified cells may benefit considerably from exposure to this information.

If I were to recommend, however, that a library acquire this very expensive volume, it is the article by Lindenmann that would induce me to do so. Lindenmann considers the various steps that led to general acceptance of the idea that a host-coded component, a carbohydrate moiety, is incorporated in budding influenza viruses. He uses this as a format to discuss both how science is done, and how credit is given (or denied) for what is done. The article is extremely readable, being written with some humour and considerable insight. This is recommended reading for students of virology, immunology and of the philosophy of science.

The book is well produced. Unfortunately, it has one major defect for those of us who read everything backwards. Individual articles are only identified at the beginning of each chapter. Printing of the authors names and/or a short title at the top of facing pages would make future volumes much more accessible for rapid reference.

P. C. Doherty

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Mammalian cell membranes

Mammalian Cell Membranes. Vol. 4: Membranes and Cellular Functions. Pp. 262. Vol. 5: Responses of Plasma Membranes. Pp. 260. Edited by G. A. Jamieson and D. M. Robinson. (Butterworths: London and Boston, Massachusetts, 1977.) £18 each volume.

WITH these books, Jamieson and Robinson close the circle of their edited series on the structure and function of mammalian cell membranes. Volume 4 is concerned with ultrastructural, biochemical and physiological aspects. In the first chapter Deamer provides a brief account of freeze-etch images and an interpretation of intramembraneous particles. This is a useful companion to later discussions of the permeability of membranes by De Pont and Bonting, and of membrane action potentials by Carpenter.

The bulk of Volume 4 is concerned with functional aspects of cell surface membranes and receptor theory. The antigenicity of cell surfaces is well covered by Weiss and Klavins—in particular, the section on the structural relationships between CEA and blood group substances—although I was startled to see references to Feizi *et al.* (1900) on pp. 74 and 95 in view of the sensationally youthful appearance of Dr Ten Feizi of the Northwick Park Clinical Research Centre.

Surface immunoglobulins and the controversy of the T-cell receptor are dealt with admirably by Kern, and Edwards provides a succinct account of current thinking in cell adhesion. Ashwell summarises the present belief of many concerning the role of surface carbohydrates in recognition phenomena, including some of those appearing in Edwards' chapter. In addition to describing briefly his own classical experiments, Ashwell reminds us of the pioneering and still provocative work of the Gasics in the related area of cell surface carbohydrates and host responses to tumours.

Volume 5 is concerned with the way in which the external environment affects the cell surface membrane, and *vice versa*. The environment in the most fundamental sense includes the effects of hydrostatic pressure (Zimmerman and Zimmerman), water structure (Meryman) and radiations (Patrick) on membranes. At the other extreme of specificity, Birnbaumer *et al.* make interesting sense of the huge literature on receptor coupling with adenylyl cyclase, and on the regulation of the coupling by nucleotides. Strangely enough, the action of cholera toxin on membranes is not

mentioned as a 'model' of hormone interactions with target cells.

In many biological processes, remodelling of cell surface membranes must occur. Several examples are considered in this volume, including endocytosis by Stockem, membrane fusion by Barski, membrane changes accompanying normal cell growth by Knox and Pasternak, and the senescent process by Robert. The best characterised of all remodelling processes, accompanying viral infection, is excellently treated by Klenk. The basic mechanisms by which membrane remodelling occurs is still a matter of speculation, although Scanlin and Glick clearly define the biochemical principles and dynamic nature of membrane turnover

and discuss the concept of membrane flow.

I have now had the opportunity to read or browse through all five books of the series on *Mammalian Cell Membranes* and no doubt shall continue to profit from the immense amount of information therein for many years. The editors are to be congratulated on the success of their project, and they and their colleagues deserve our gratitude for providing a solid platform from which to view the rapidly changing membrane scene.

R. C. Hughes

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Strange compendium

Handbook of Unusual Natural Phenomena. By William R. Corliss. Pp. 542. (The Sourcebook Project; Glen Arm, Maryland, 1977.) \$14.95.

WILLIAM CORLISS has been producing his 'sourcebooks' of such strange phenomena as ball lightning, plagues of frogs and miscellaneous phenomena associated with earthquakes since 1974. In the past, these have taken the form of loose-leaf folders containing synoptical accounts of observations reported in a wide variety of journals (with *Nature* prominent among them) and over a long period (back into the nineteenth century). The present 'handbook' presents the mixture as before but in more substantial and permanent form, as a bound volume of more than 500 pages, full of fascinating morsels.

The entries range in length from a couple of lines to a page or more plus diagrams, and cover in this volume

phenomena grouped together as luminous phenomena, optical and radio anomalies, unusual weather phenomena, mysterious natural sounds, strange phenomena of earthquakes, phenomena of the hydrosphere and falling material. For anyone suffering from the delusion that science has reached a comforting state of explaining just about everything, the book may provide a few shocks; for the less sanguine among us, here is a delightful source of bed-time reading, full of anecdotes with which to amaze and amuse colleagues the next day. And, who knows, by reviving interest in such curiosities as the shower of sand eels reported by A. Meek in *Nature* (102, 46; 1918), Corliss may well encourage the development of an improved scientific understanding of some unusual natural phenomena.

John Gribbin

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Beat the machine

Advances in Computer Chess. Edited by M. R. B. Clarke. Pp. 118. (Edinburgh University: Edinburgh, 1977.) £3.50.

IT is now many years since the electronic computer greatly exceeded the human brain in terms of speed and accuracy in calculation. But even in applications to well defined, finite games, such as chess, where this quality is at a premium, computers cannot match the performance of the more able human players.

This human advantage can largely be attributed to the possession of an ill-defined capability known as 'common sense', a concept unknown to computers. In the situation of the chess game, its activities are manifested in the human's ability to reject most of

the moves available to him as unworthy of analysis, and to analyse only those which are 'sensible'.

This book, consisting of a set of articles from a symposium held in Oxford in 1975, deals with the problem of programming 'common sense' into a computer. In the chess context, this amounts principally to techniques of search reduction at the tactical level, and rudimentary 'planning' at the strategic level. A short article on the mathematical theory of search techniques, and several examples of the success (or lack of it) of practical programmes, complete a collection which should be worthwhile reading for those interested in the problems of machine intelligence.

N. J. Holloway

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Narrow gap semiconductors

Semimetals and Narrow-Bandgap Semiconductors. By D. R. Lovett. Pp. viii + 256. (Pion: London, 1977.) £8.50; \$17.50.

AT FIRST GLANCE this book has a pleasing appearance with many clear diagrams and graphs, and a more detailed examination has convinced me that the breadth and depth of coverage should make it a very useful book for those entering the field of electrical properties and applications of narrow gap semiconductors.

The book is basically in two parts. In the first part, there is an excellent and concise introduction to phase diagrams and crystal growing techniques, clearly outlining the many pitfalls and problems which would confront anyone trying to prepare pure and homogeneous materials. The chapter on crystal symmetry and band properties is probably the weakest part of the book, for it is

so concise as to be practically of no help to anyone except those already initiated in the art. The electrical properties is well treated and is somewhat similar to Putley's well-known book *Hall Effect and Related Phenomena*. There is also a chapter briefly reviewing narrow band gap devices.

The real interest of the author is expressed in the second half of the book, dealing with the much more detailed aspects of particular materials, ranging from bismuth-antimony alloys to selenides, tellurides and arsenides. This latter part of the book would certainly be of very considerable use as a source of information for both the researcher as well as those interested in the application of narrow band semiconductors.

At £8.50 this book is certainly value for money for those on the periphery of this field.

M. E. Ertl

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Analysing pure substances

Spectrochemical Analysis of Pure Substances. Edited by Kh. I. Zil'berstein. Translated by J. H. Dixon. Pp. 435. (Plenum: London and New York, 1977.) £27.

THIS English translation of the (1971) Russian edition, stated by the publishers to be thoroughly revised and up-to-date, contains 1,497 references, over half to Russian work, but less than ten after 1971. A vast amount of information on trace analysis is given, most of it in an uncritical review form of Russian and Eastern European work.

In Part I, the first three chapters give excellent, but over-lengthy and unbalanced, sections on evaluating limits of detection, and their improvement by optimising parameters of spectral resolution. Reference to spectrographic equipment by symbol only would have been of more value if a table had been given listing their main features such as focal length. In chapter 4 a long description is given of arc sources which, although biased towards Russian work, describes in detail the various distribution parameters across electrode gaps of d. c. and a. c. arcs, with anode and cathode excitation, and the resulting line-to-background ratios. Techniques for introducing samples into the arc are reviewed and methods of stabilisation, such as the use of external magnetic fields and various forms of constricted arcs are discussed. Other sources are covered in less detail, but the glow discharge is noticeably absent.

Part 2 describes preliminary concentration methods, starting with a rather cumbersome mathematical section on the expression of the concentration factor, followed by more useful sections on concentration by volatilisation, sublimation, crystallisation, zone melting, solvent extraction, ion exchange, coprecipitation and electrodeposition.

Part 3 deals with practical problems including sampling, grinding, laboratory design, purification of reagents and carbon electrodes, and more briefly with the preparation of synthetic standards. The final section, on practical methods of analysing pure substances, is a ten-page list of sample types, noting the trace elements determined, and giving references in the bibliography.

The contents of the book and its translation are excellent. It is full of valuable information, but this can only be found by thorough study, as the index is totally inadequate. I can foresee two types of reader: the spectroscopist, who may obtain some new lines and those who wish to be made aware of Eastern-European work. Both would be justified in purchasing this book. Readers should note, however, that some of the Russian claims to priority are unjustified according to the record of world literature.

T. S. West

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Endocrinology yearbook

The Year in Endocrinology, 1975-1976. Edited by Sidney H. Ingbar. Pp. xviii + 327. (Plenum Medical: New York and London, 1976.) \$27.

'YEARBOOKS' come in many shapes and sizes. Probably the commonest format is the upgraded textbook in which a series of chapters, whose headings embrace non-selectively the whole of the subject under consideration, seek to convey to the reader the principal advances which have taken place since the previous edition. Often, each chapter will consist of summaries of key papers accompanied by editorial comment. The contrary approach is the presentation of a series of highly selective reviews by eminent authorities, usually with a heavy bias towards the work of their own groups. The present volume falls somewhere between the two. Nine chapters by individual authors review the principal endocrine systems except those of the foeto-placental unit and the gut. Most are comprehensive, but there are occasional notable exclusions: for example, the section on neuroendocrinology makes no mention of the posterior pituitary gland, whereas another concentrates on calcitonin and ignores parathyroid hormone. Finally, there are two special essays on ectopic hormone secretion and the mechanisms of steroid hormone action.

The general standard of the individual chapters is high and as a review of the state of the art for readers in 1977 there is no doubt that this is a useful book. The transatlantic flavour, however, is noticeable by the absence of comment on important therapeutic advances. For example, the use of ergot derivatives in the treatment of hyperprolactinaemia is dismissed in a few lines and a single minor reference. This is surprising since the use of Bromocriptine is probably the single most important development in endocrine management of the 1970s, and the bulk of the significant papers on this subject appeared in the time period in which this book purports to cover. Far more space is devoted to the use of the gonadotrophin-releasing hormone, despite the fact that it is unlikely this material will ever be of great practical value. These comments apart, the book can be commended to the practising endocrinologist.

T. Chard

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Recent scientific and technical books

Biology

- JOHNSON, Martin H. (edited by). *Development in Mammals*, Vol. 1. Pp.vii + 390. ISBN-0-7204-0631-5. (Amsterdam, New York and Oxford: North-Holland Publishing Company, 1977.) np.
- JOHNSON, Martin (edited by). *Development in Mammals*, Vol. 2. Pp.vii + 241. ISBN-0-7204-0646-3. (Amsterdam, New York and Oxford: North-Holland Publishing Company, 1977.) Dfl. 72; \$29.50.
- JUNGREIS, Arthur M., HODGES, Thomas K., KLEINZELLER, Arnold, and SCHULTZ, Stanley G. (edited by). *Water Relations in Membrane Transport in Plants and Animals*. Pp.xiv + 393. ISBN-0-12-392050-7. (New York and London: Academic Press, a Subsidiary of Harcourt Brace Jovanovich, Publishers, 1977.) \$18; £12.80.
- KAHRIG, Erwin, and BESSERDICH, Hartmut. *Dissipative Strukturen: Eine Einführung in den Problembereich und die Erscheinungsformen. (Fortschritte der Experimentellen und Theoretischen Biophysik, Band 21.)* Pp.116. (Leipzig: Veb Georg Thieme, 1977.) DDR 29 Mark.
- KERKUT, G. A., and PHILLIS, J. W. (edited by). *Progress in Neurobiology*, Vol. 7. Pp.vii + 398. ISBN-0-08-020321-3. (Oxford and New York: Pergamon Press, 1977.) £27.
- KLUGE, Arnolds G. In collaboration with FRYE, B. E. *et al.* *Chordate Structure and Function*. Second edition. Pp.x + 628. ISBN-0-02-364800-7. (New York: Macmillan Publishing Co., Inc.; London: Collier Macmillan Publishers, 1977.) £12.
- KORN, Edward D. (edited by). *Methods in Membrane Biology*, Vol. 8. Pp.xix + 368. ISBN-0-306-36808-0. (v. 8). (New York and London: Plenum Press, 1977.) \$39.50.
- KREIER, Julius P. (edited by). *Parasitic Protozoa*. Vol. 1: Taxonomy, Kinetoplastids, and Flagellates of Fish. Pp.xv + 441. ISBN-0-12-426001-2. (New York and London: Academic Press, a Subsidiary of Harcourt Brace Jovanovich, Publishers, 1977.) \$35; £24.85.
- KUMAR, H. D., and SINGH, H. N. *Plant Metabolism*. Pp.vii + 302. (New Delhi and Madras: Affiliated East-West Press, Pvt. Ltd., 1976.) Rs. 18.
- LAGLER, Karl F., BARDACH, John E., MILLER, Robert M., and PASSINO, Dora R. May. *Ichthyology*. Second edition. Pp.xv + 506. ISBN-0-471-51166-8. (New York and London: John Wiley and Sons, 1977.) £13.50; \$23.50.
- LOVTRUP, Søren. *The Phylogeny of Vertebrata*. Pp. xii + 330. ISBN-0-471-99412-X. (London and New York: Wiley-Interscience, John Wiley and Sons, 1977.) £15; \$29.50.
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- MALICKY, Hans (herausgegeben von). *Verhandlungen des Sechsten Internationalen Symposiums über Entomofaunistik in Mitteleuropa, Lunz am See, Österreich, 1-6 September 1975*. Pp.375. ISBN-90-6193-559-8. (Den Haag: Verlag Dr. W. Junk, 1977.) Dutch guilders 95.
- MARKHAM, Roy, and HORNE, R. W. (edited by). *Structure-Function Relationships of Proteins*. (Proceedings of the Third John Innes Symposium held in Norwich, July 1976.) Pp.vii + 204. ISBN-0-7204-0613-7. (Amsterdam, New York and Oxford: North-Holland Publishing Company, 1976.) Dfl. 78.50; \$31.95.
- NAPIER, J. R. *Primates and Their Adaptations*. (Carolina Biology Readers, No. 28.) Pp.16. ISBN-0-89278-228-5. (Burlington, North Carolina, Scientific Publications Division, Carolina Biological Supply Company, 1977.) np.
- NEZLIN, R. S. *Structure and Biosynthesis of Antibodies*. Translated from Russian by Basil Haigh. Translated, edited by Felix Haurowitz. (Studies in Soviet Science: Life Sciences.) Pp.xv + 367. ISBN-0-306-10921-2. (New York and London: Consultants Bureau, 1977.) £42.
- NORTHCOTE, D. H. (edited by). *Plant Biochemistry II*. (International Review of Biochemistry, Vol. 13.) Pp.ix + 262. ISBN-0-8391-1077-4. (Baltimore: University Park Press; Lancaster: MTP Press, Ltd., 1977.) £21.25.
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- OLNEY, P. J. S. (edited by). *International Zoo Yearbook*, Vol. 17. Pp.x + 392 + 60 plates. ISBN-0074-9664. (London: The Zoological Society of London, 1977.) Hardbound £12; \$24. Paperback £9; \$18.
- PIELOU, E. C. *Mathematical Ecology*. Pp.x + 385. ISBN-0-471-01993-3. (New York and London: Wiley-Interscience, John Wiley and Sons, 1977.) £13; \$22.45.
- PRESCOTT, David M. (edited by). *Methods in Cell Biology*, Vol. 15. Pp.xix + 490. ISBN-0-12-564115-X. (New York and London: Academic Press, a Subsidiary of Harcourt Brace Jovanovich, Publishers, 1977.) np.
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- READER, John, and CROZE, Harvey. *Pyramids of Life: An Investigation of Nature's Fearful Symmetry*. Pp.222. ISBN-0-00-219004-4. (London: William Collins Sons and Co., Ltd., 1977.) £6.95.
- REES, H. H. *Insect Biochemistry*. (Outline Studies in Biology.) Pp.64. ISBN-0-412-13130-7. (London: Chapman and Hall, 1977. Distributed in the USA by Halsted Press, a Division of John Wiley and Sons, Inc., New York.) £1.50.
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- TS'O, Paul O. P. (edited by). *The Molecular Biology of the Mammalian Genetic Apparatus*, Vol. 1. Pp.xx + 444. ISBN-0-7204-0625-0. (Amsterdam, New York and Oxford: North-Holland Publishing Company, 1977.) Dfl. 112.50; \$46.
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- WILSON, Edward O., EISNER, Thomas, BRIGGS, Winslow R., DICKERSON, Richard E., METZENBERG, Robert L., O'BRIEN, Richard D., SUSMAN, Millard, and BOGGS, William E. *Life: Cells, Organisms, Populations*. Pp.viii + 546. ISBN-0-87893-943-1. (Sunderland, Mass.: Sinauer Associated, Inc., 1977. Distributed in UK by W. H. Freeman and Co., Ltd., Reading.) £10.20.
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- WOODWARD, Dow O., and WOODWARD, Val W. *Concepts of Molecular Genetics: Information Flow in Genetics and Evolution*. (McGraw-Hill Series in Population Biology.) Pp.xiii + 418. (New York and London: McGraw-Hill Book Company, 1977.) £11.20.

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- CHRISPEELS, Maarten J. *Plants, Food and People*. (A Series of Books in Biology.) Pp.278. ISBN-0-7167-0377-7. (San Francisco and Reading: W. H. Freeman and Company, 1977.) Hardcover £9.60; Softcover £4.80.
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- CROSIGNANI, P. G., and ROBYN, C. (edited by). *Prolactin and Human Reproduction*. (Proceedings of the Sero Symposium, Vol. 11.) Pp.vii + 305. ISBN-0-12-198345-5. (London and New York: Academic Press, a Subsidiary of Harcourt Brace Jovanovich, Publishers, 1977.) £10.50; \$20.50.
- CUATRECASAS, P., and GREAVES, M. F. (edited by). *Receptors and Recognition*. Series A. Pp. viii + 166. ISBN-0-412-14320-8. (London: Chapman and Hall; New York: Halstead Press, a Division of John Wiley and Sons, Inc., 1977.) Cased £10; Paperback £6.
- CURRY, Stephen H. *Drug Disposition and Pharmacokinetics, with a Consideration of Pharmacological and Clinical Relationships*. Pp.xi + 275. Second edition. ISBN-0-632-00476-2. (Oxford and London: Blackwell Scientific Publications, 1977.) £6.25.
- DALY, John. *Cyclic Nucleotides in the Nervous System*. Pp. xiv + 401. ISBN-0-306-30971-8. (New York and London: Plenum Press, 1977.) \$39.
- DAY, Charles E., and LEVY, Robert S. (edited by). *Low Density Lipoproteins*. Pp.xx + 445. ISBN-0-306-30934-3. (New York and London: Plenum Press, 1976.) \$47.40.
- DAY, Nooribi, and GOOD, Robert A. (edited by). *Biological Amplification Systems in Immunology*. (Comprehensive Immunology, Vol. 2.) Pp.xv + 325. ISBN-0-306-33102-0. (New York and London: Plenum Medical Book Company, 1977.) np.
- DRUCKER-COLIN, René R., and MCGAUGH, James (edited by). *Neurobiology of Sleep and Memory*. (Proceedings of a Conference held in Mexico City in March, 1975.) Pp.xiii + 456. ISBN-0-12-222350-0. (New York and London: Academic Press, a Subsidiary of Harcourt Brace Jovanovich, Publishers, 1977.) \$19.50; £13.85.
- DUNCAN, W. A. M., and LEONARD, B. J. (edited by). *Clinical Toxicology*. (Proceedings of the Meeting held at Edinburgh, June 1976.) (Proceedings of the European Society of Toxicology, Vol. XVIII, 1977.) Pp.ix + 348. ISBN-0-444-15248-2. (Amsterdam and Oxford: Excerpta Medica, 1977. Distributed in the USA and Canada by Elsevier North-Holland, Inc., New York.) Dfl. 87.50; \$35.75.

- DUNCAN, Wm., and NIAS, A. H. W. *Clinical Radiobiology*. Pp.226. ISBN-0-443-01147-8. (Edinburgh, London and New York: Churchill Livingstone, 1977.) £9.50.
- ELIAS, P. S., and COHEN, A. J. (edited by). *Radiation Chemistry of Major Food Components: Its Relevance to the Assessment of the Wholesomeness of Irradiated Foods*. Pp.xii+220. ISBN-0-444-41587-4. (Amsterdam, Oxford and New York: Elsevier Scientific Publishing Company, 1977.) Dfl. 63; \$25.75.
- ELLINWOOD, Jr., Everett H. (edited by). *Cocaine and Other Stimulants*. (Advances in Behavioral Biology, Vol. 21.) Pp.x+721. ISBN-0-306-37921-X. (New York and London: Plenum Press, 1977.) \$59.40.
- EPSTEIN, T., Scarlett, and JACKSON, Darrell (edited by). *The Feasibility of Fertility Planning: Micro Perspectives*. Pp.viii+244. ISBN-0-08-021837-7. (Oxford and New York: Pergamon Press, 1977.) Hardcover £12.50; Flexicover £4.
- ESSMAN, Walter B. (edited by). *Serotonin in Health and Disease*. Vol. 4: *Clinical Correlates*. Pp.444. ISBN-0-89335-003-6 (v. 4). (New York: S.P. Books Division of Spectrum Publications, Inc., 1977.) \$37.50.
- FERTILIZER USE AND PROTEIN PRODUCTION. (Proceedings of the 11th Colloquium of the International Potash Institute held in Rzonne-Bornholm, Denmark, 1975.) Pp.307. (Worblaufen-Bern: International Potash Institute, 1975.) Sw. fr. 28; \$11.70.
- THE FETUS AND BIRTH. (Ciba Foundation Symposium 47, New Series.) Pp.viii+481. ISBN-0-444-15255-5. (Amsterdam, Oxford and New York: Elsevier/Excerpta Medica/North-Holland, 1977.) Dfl. 98; \$39.95.
- DeFRANCE, Jon F. (edited by). *The Septal Nuclei*. (Advances in Behavioral Biology, Vol. 20.) Pp.xi+539. ISBN-0-306-37920-1. (New York and London: Plenum Press, 1976.) \$47.40.
- FRANKEL, R., and GALUN, E. *Pollination Mechanisms, Reproduction and Plant Breeding*. (Monographs on Theoretical and Applied Genetics, Vol. 2.) Pp.xi+281. ISBN-3-540-07934-3. (Berlin and New York: Springer-Verlag, 1977.) DM 60; \$26.40.
- FREINKEL, Norbert (edited by). *The Year in Metabolism, 1975-1976*. Pp.xxi+353. ISBN-0-306-32001-0. (New York and London: Plenum Medical Book Company, 1976.) \$27.
- FREY, R., NAGEL, E., and SAFAR, P. (edited by). *Mobile Intensive Care Units: Advanced Emergency Care Delivery Systems*. (Anaesthesiology and Resuscitation/Anaesthesiologie und Wiederbelebung/Anesthésiologie et Réanimation, Vol. 95.) Pp.xv+271. ISBN-3-540-07561-5. (Berlin and New York: Springer-Verlag, 1976.) DM 48; \$19.70.
- FRIGERO, Alberto, and GHISALBERTI, Emilio L. (edited by). *Mass Spectrometry in Drug Metabolism*. Pp.xii+532. ISBN-0-306-31018-X. (New York and London: Plenum Press, 1977.) np.
- GLICK, David, and ROSENBAUM, Robert M. (edited by). *Techniques of Biochemical and Biophysical Morphology*. Vol. 3. Pp.ix+214. ISBN-0-471-02219-5. (New York and London: Wiley-Interscience, a Division of John Wiley and Sons, Inc., 1977.) £17.25; \$29.
- GOLTERMAN, H. L. (edited by). *Interactions Between Sediments and Fresh Water*. (Proceedings of an International Symposium held at Amsterdam, The Netherlands, September 6-10, 1976.) Pp.473. ISBN-90-06193-563-6. (The Hague: Dr. W. Junk B.V. Publishers; Wageningen: Centre for Agricultural Publishing and Documentation, 1977.) Dutch guilders 65.
- GRAYSON, John, and ZINGG, Walter (edited by). *Microcirculation*. Vol. 2: *Transport Mechanisms; Disease States*. Pp.xvii+361. ISBN-0-306-37098-0 (v. 2). (New York and London: Plenum Press, 1976.) \$39.
- GREATHEAD, D. J. (edited by). *A Review of Biological Control in Western and Southern Europe*. (Technical Communication No. 7.) Pp.182. ISBN-0-85198-369-3. (Farnham Royal, Slough: Commonwealth Agricultural Bureaux, 1976.) np.
- GROTE, Jürgen, RENEAU, Daniel, and THEWS, Gerhard (edited by). *Oxygen Transport to Tissue-II*. (Advances in Experimental Medicine and Biology, Vol. 75.) Pp.xviii+781. ISBN-0-306-39075-2. (New York and London: Plenum Press, 1976.) \$59.40.
- HAMILTON, Leonard W. *Basic Limbic System Anatomy of the Rat*. Pp.xvii+150. ISBN-0-306-30925-4. (New York and London: Plenum Press, 1976.) \$23.40.
- HANNA, Michael G., and RAPP, Fred (edited by). *Contemporary Topics in Immunobiology*. Vol. 6: *Immunobiology of Oncogenic Viruses*. Pp.xiii+289. ISBN-0-306-37806-X. (New York and London: Plenum Press, 1977.) \$33.
- HARDIN, Garrett, and BADEN, John (edited by). *Managing the Commons*. Pp.xii+294. ISBN-0-7167-0476-5. (San Francisco and Reading: W. H. Freeman and Company, 1977.) £5.10.
- HARRISON, R. G., and de BOER, C. H. *Sex and Infertility*. (Monographs for Students of Medicine.) Pp.xii+122. ISBN-0-12-327850-3. (London and New York: Academic Press, a Subsidiary of Harcourt Brace Jovanovich, Publishers, 1977.) £2.80; \$5.50.
- HERING, Ewald. *The Theory of Binocular Vision*. Edited by Brice Bridgeman and Lawrence Stark. Translation and Introduction by Brice Bridgeman. Commentary by Lawrence Stark. Pp.218. ISBN-0-306-31016-3. (New York and London: Plenum Press, 1977.) \$29.40.
- IMMUNOLOGY OF THE GUT. (Ciba Foundation Symposium, No. 46, New Series.) Pp.viii+376. ISBN-0-444-15246-6. (Amsterdam, Oxford and New York: Elsevier/Excerpta Medica/North-Holland, 1977.) Dfl. 70; \$28.75.
- IMPERATO, Pascal James. *African Folk Medicine: Practices and Beliefs of the Bambara and Other Peoples*. Pp.xvii+251. ISBN-0-912752-08-4. (Baltimore, Md.: York Press, Inc., 1977.) \$16.
- INGBAR, Sidney H. (edited by). *The Year in Endocrinology, 1975-1976*. Pp.xviii+327. ISBN-0-306-32101-7. (New York and London: Plenum Medical Book Company, 1976.) \$27.
- JACKSON, J. I. *Climate, Water and Agriculture in the Tropics*. Pp.xii+248. ISBN-0-582-48529-0. (London and New York: Longman, 1977.) £3.75.
- JOLLOU, David J., KOCIS, James, SNYDER, Robert, and VAINIO, Harri (edited by). *In association with SAUKKONEN, Jussi, WITMER, Charlotte, and WALLE, Thomas. Biological Ractive Intermediates: Formation, Toxicity, and Inactivation*. Pp.xi+514. ISBN-0-306-30970-X. (New York and London: Plenum Press, 1977.) \$59.40.
- KANE, Robert L. (edited by). *The Behavioral Sciences and Preventive Medicine: Opportunities and Dilemmas*. (A Conference sponsored by the John E. Fogarty International Center for Advanced Study in the Health Sciences, and the Association of Teachers of Preventive Medicine, National Institutes of Health, Bethesda, Md., December 5 and 6, 1974.) Pp.xvii+261. (Washington, DC: US Government Printing Office, 1977.) \$6.00.
- KILEY, WORTHINGTON, M. *Behavioural Problems of Farm Animals*. Pp.vii+134. ISBN-0-85362-163-2. (Stocksfield, London and Boston, Mass.: Oriel Press Ltd. (Routledge and Kegan Paul, Ltd.), 1977.) £4.
- KISSIN, Benjamin, and BEGLEITER, Henri (edited by). *Treatment and Rehabilitation of the Chronic Alcoholic*. (The Biology of Alcoholism, Vol. 5.) Pp.xxv+631. ISBN-0-306-37115-4 (v. 5). \$47.40.
- KLEIN, George, and WEINHOUSE, Sidney (edited by). *Advances in Cancer Research*. Vol. 25. Pp.ix+401. ISBN-0-12-006625-4. (New York and London: Academic Press, a Subsidiary of Harcourt Brace Jovanovich, Publishers, 1977.) \$29.50; £20.95.
- KLINGMULLER, W. *Genmanipulation und Gentherapie*. Pp.viii+345. ISBN-3-540-07903-3. (Berlin and New York: Springer-Verlag, 1976.) DM 38; \$15.60.
- KNOWLES, John H. (edited by). *Doing Better and Feeling Worse: Health in the United States*. Pp.278. (New York: W. W. Norton and Company, 1977.) Cloth \$9.95; Paper \$3.95.
- KRAMER, M., and LAUTERBACH, F. (edited by). *Intestinal Permeation*. Proceedings of the Fourth Workshop Conference Hoechst, Schloss Reisenburg, 19-22 October, 1975.) (Workshop Conferences Hoechst, Vol. 4.) Pp.xii+449. ISBN-0-444-15225-3. (Amsterdam and Oxford: Excerpta Medica, 1977.) Distributed in USA and Canada by Elsevier North-Holland, Inc., New York.) Dfl. 160; \$65.50.
- KRAUSE, W. (edited by). *Application of Vegetation Science to Grassland Husbandry*. (Handbook of Vegetation Science, Part 13.) Pp.xx+535. ISBN-90-6193-194-0. (The Hague: Dr. W. Junk B.V. Publishers, 1977.) Dutch guilders 150.
- LURIYA, E. A. *Hematopoietic and Lymphoid Tissue in Culture*. Translated from Russian by Basil Haigh. (Studies in Soviet Science.) Pp.xiii+181. ISBN-0-306-10934-4. (New York and London: Consultants Bureau a Division of Plenum Publishing Corporation, 1977.) \$42.
- LAL, Harbans (edited by). *Discriminative Stimulus Properties of Drugs*. (Advances in Behavioral Biology, Vol. 22.) Pp.xii+239. ISBN-0-306-37922-8. (New York and London: Plenum Press, 1977.) \$30.
- LANCE, E. M., MEDAWAR, P. B., and SIMPSON, E. (edited by). *An Introduction to Immunology*. Pp.xii+158. ISBN-0-7045-0209-7. (London: Wildwood House, Ltd., 1977.) £4.95 net.
- LAUFFER, Max A., BANG, Frederick B., MARAMOROSCH, Karl, and SMITH, Kenneth M. *Advances in Virus Research*. Vol. 21. Pp.vii+409. ISBN-0-12-039821-4. (New York and London: Academic Press, a Subsidiary of Harcourt Brace Jovanovich, Publishers, 1977.) \$29.50; £20.95.
- LEE, Peter A., PLOTNICK, Leslie P., KOWARSKI, A. A., and MIGEON, Claude J. (edited by). *Congenital Adrenal Hyperplasia*. Pp.xvii+532. ISBN-0-8391-0974-1. (Baltimore and Tokyo: University Park Press; Lancaster: MTP Press, Ltd., 1977.) £26.95.
- LOOR, F., and ROELANTS, G. E. (edited by). *B and T Cells in Immune Recognition*. Pp.viii+504. ISBN-0-471-99438-3. (London and New York: Wiley-Interscience, John Wiley and Sons, 1977.) £18.50; \$37.50.
- MAKINODAN, Takashi, and YUNIS, Edmond (edited by). *Immunology and Aging*. (Comprehensive Immunology, Vol. 1.) Pp.xv+208. ISBN-0-306-33101-2. (New York and London: Plenum Medical Book Company, 1977.) \$27.
- MADDEN, J. S., WALKER, Robin, and KENYON, W. H. (edited by). *Alcoholism and Drug Dependence: a Multidisciplinary Approach*. Pp.xix+479. ISBN-0-306-31019-8. (New York and London: Plenum Press, 1977.) \$47.40.
- MARCHALONIS, John J. (edited by). *The Lymphocyte: Structure and Function*. Part 1. (Immunology Series, 5.) Pp.xi+369. ISBN-0-8247-6418-8. (New York and Basel: Marcel Dekker, Inc., 1977.) SFr. 123.
- MARTIN, Hubert, and WORTHING, Charles R. (edited by). *Insecticide and Fungicide Handbook for Crop Protection*. (Issued by the British Crop Protection Council.) Pp.xvi+427. Fifth edition. ISBN-0-632-00109-7. (Oxford and London: Blackwell Scientific Publications, 1976.) Distributed in USA and Canada by J. B. Lippincott Company.) £12.50.
- MARTINEZ-MALDONADO, Manuel (edited by). *Methods in Pharmacology*. Vol. 4A: *Renal Pharmacology*. Pp.x+387. ISBN-0-306-35264-8. (New York and London: Plenum Press, 1976.) \$47.40.
- MARZULLI, Francis N., and MAIBACH, Howard I. (edited by). *Dermatotoxicology and Pharmacology*. (Advances in Modern Toxicology, Vol. 4.) Pp.xviii+567. ISBN-0-470-99063-5. (Washington: Hemisphere Publishing Corporation, 1977.) Distributed by Halsted Press, a Division of John Wiley and Sons, Inc., New York and Chichester.) £26; \$44.
- MASSRY, Shaul G. (edited by). *Kidney in Systemic Diseases*. (Contributions to Nephrology, Vol. 7.) Pp. viii+308. ISBN-3-8055-2445-5. (Basel, London and New York: S. Karger, 1977.) SFr./DM 98; \$37.75.
- MOHR, U., SCHMAHL, D., and TOMATIS, L. (edited by). DAVID, W. (technical editor). *Air Pollution and Cancer in Man*. (Proceedings of the Second Hanover International Carcinogenesis Meeting held in Hanover, 22-24 October, 1975.) Pp. xvii+331. ISBN-92-832-1116-2. (Lyon: International Agency for Research on Cancer, 1977.) Distributed by WHO, Geneva.) SFr. 90; \$35.
- MORELL, Pierre (edited by). *Myelin*. Pp. xxi+531. ISBN-0-306-30965-3. (New York and London: Plenum Press, 1977.) \$47.40.
- MUNOZ, J. J., and BERGMAN, R. K. *Bordetella pertussis: Immunological and Other Biological Activities*. (Immunology Series, Vol. 4.) Pp.235. ISBN-0-8247-6507-9. (New York and Basel: Marcel Dekker, Inc., 1977.) SFr. 82.
- NAGAICH, B. B. (edited by). *Recent Technology in Potato Improvement and Production*. (Lectures delivered during the Summer Institute, held at the Central Potato Research Institute, Simla, from June 21 to July 20, 1976.) Pp.iv+310. (Simla: Central Potato Research Institute, Indian Council of Agricultural Research, 1977.) np.
- NAIR, P. P., and KRITCHEVSKY, David (edited by). *The Bile Acids: Chemistry, Physiology, and Metabolism*. Vol. 3: *Pathophysiology*. Pp.xiii+229. ISBN-0-306-37133-2. (New York and London: Plenum Press, 1976.) \$27.
- OXFORD, J. S., DRASAR, F. A., and WILLIAMS, J. D. (edited by). *Chemotherapy of Herpes Simplex Virus Infections*. (Reprinted from Supplement A of the *Journal of Antimicrobial Chemotherapy*, Vol. 3, March 1977.) Pp.vii+141. (London and New York: Academic Press, 1977.) £4.80; \$9.35.
- PAOLETTI, R., and SHERRY, S. (edited by). *Thrombosis and Urokinase*. (Proceedings of the Sero Conference, Vol. 9.) Pp.viii+257. ISBN-0-12-544960-7. (London and New York: Academic Press, a Subsidiary of Harcourt Brace Jovanovich, Publishers, 1977.) £8.20; \$16.
- PASQUALINI, Jorge E. (edited by). *Receptors and Mechanism of Action of Steroid Hormones, Part 2. Modern Pharmacology-Toxicology: a Series of Monographs and Textbooks*. Vol. 8.) Pp.ix+311-736. ISBN-0-8247-6440-4. (New York and Basel: Marcel Dekker, Inc., 1977.) SFr. 130.
- PETTIT, George R. *Biosynthetic Products for Cancer Chemotherapy*. Vol. 1. Pp.xii+215. ISBN-0-306-37687-3 (v.1). (New York and London: Plenum Press, 1977.) \$23.40.
- PORTER, R. R., and ADA, G. L. (edited by). *Contemporary Topics in Molecular Immunology*. Vol. 6. Pp.xiii+252. ISBN-0-306-36106-X. (New York and London: Plenum Press, 1977.) \$30.
- PRASAD, M. R. N., and KUMAR, T. C. Anand (edited by). *Use of Non-Human Primates in Biomedical Research*. (International Symposium held in New Delhi, India, November 3-8, 1975.) Pp.426. (New Delhi: Indian National Science Academy, 1977.) np.
- PRESTON, Thomas A. *Coronary Artery Surgery: a Critical Review*. Pp.xi+266. ISBN-0-89004-165-2. (New York: Raven Press, 1977.) \$15.
- PURINE AND PYRIMIDINE METABOLISM. (Ciba Foundation Symposium 48, New Series.) Pp.xi+369. ISBN-0-444-15256-3. (Amsterdam, Oxford and New York: Elsevier/Excerpta Medica/North-Holland, 1977.) Dfl. 76; \$30.95.
- QURASHI, M. Sayeed. *Biochemical Insect Control: Its Impact on Economy, Environment, and Natural Selection*. Pp.viii+280. ISBN-0-471-70275-7. (New York and London: Wiley/Interscience, John Wiley and Sons, 1977.) £14.25; \$24.50.
- RAJKA, E., and KOROSSY, S. (edited by). *Immunological Aspects of Allergy and Allergic Diseases*. Vol. 6: *Antigen-Antibody Reactions in Different Organs*. Pp.vii+343. ISBN-0-306-37756-X. (London and New York: Plenum Press; Budapest: Akadémiai Kiadó, 1976.) \$42.
- RATCLIFFE, Derek (edited by). *A Nature Conservation Review: The Selection of Biological Sites of National Importance to Nature Conservation in Britain*. Vol. 1. Pp.xvi+401. ISBN-0-521-21159-X. £35 net. Vol. 2. Pp.viii+320. ISBN-0-521-21403-3. £25 net. (Cambridge, London and New York: Cambridge University Press, 1977.)
- RECHCIGL, Jr., M. (edited by). *Nutrient Elements and Toxicants*. (Comparative Animal Nutrition, Vol. 2.) Pp.x+210. ISBN-3-8055-2351-3. (Basle, London and New York: S. Karger, 1977.) SFr./DM 98; \$37.75.
- REID, Robert. *My Children, My Children: Life Before and After Birth—an Account of Some Recent Developments*. Pp.186. ISBN-0-563-12857-7. (London: British Broadcasting Corporation, 1977.) £3.50.
- REINHOLD, L., HARBORNE, J. B., and SWAIN, T. (edited by). *Progress in Phytochemistry*. Vol. 4. Pp.289. ISBN-0-08-021004-X. (Oxford and New York: Pergamon Press, 1977.) £16.50.
- REIVICH, Martin, COBURN, Ronald, LAHIRI, Sukhamay, and CHANCE, Britton. *Tissue Hypoxia and Ischemia*. (Advances in Experimental Medicine and Biology, Vol. 78.) Pp.xiv+385. ISBN-0-306-39078-7. (New York and London: Plenum Press, 1977.) \$45.

announcements

On the Move

R. S. Barnes, Chief Scientist of the British Steel Corporation since 1975, and previously its Director of Research and Development, to Principal of Queen Elizabeth College, London University, on 1 April 1978.

K. G. Adiyodi, Professor of Reproductive Physiology, Calicut University, India and Secretary, International Society for Invertebrate Reproduction, to Department of Zoology, Oxford University, South Parks Road, Oxford.

C. D. Bridges, Professor of Experimental Ophthalmology at New York University Medical Center, to Professor of Ophthalmology at the Baylor College of Medicine, Houston, Texas.

Professor Y. K. Levine from Department of Physical Chemistry, University of Leeds, to the Chair of Biophysics at the University of Utrecht.

Details of changes of department, sabbaticals, where leave will be taken and so on should be sent to On the Move; there is no charge for this service.

Appointments

Sir Ieuan Maddock, previously Chief Scientist at the Department of Industry, is to succeed Magnus Pyke as Secretary of the British Association for the Advancement of Science on 7 September 1977.

R. Frosch, previously a director of the Woods Hole Oceanographic Institution, to Head of NASA.

A. J. Lee, Director of Fisheries Research at Lowestoft for the Ministry of Agriculture, Fisheries and Food, to Controller of Fisheries Research and Development for Great Britain.

J. Black, Professor of Pharmacology at University College, London, is to join the Wellcome Research Laboratories at Beckenham, Kent, as Director of Therapeutic Research. He will lead the search for new chemical compounds and their testing for activity and safety with the aim of finding new medicines for the control of the functions of the body. He expects to be working at Beckenham full-time by 1 January, 1978.

A. C. Fabian to the newly-established Radcliffe Fellowship in Astronomy, to be based at the Cambridge Institute of Astronomy where his research will

concentrate on X-ray astronomy and related optical studies.

W. D. M. Paton, Professor of Pharmacology at the University of Oxford is to succeed Sir J. McMichael as a trustee of the Wellcome Trust from 1 January 1978.

Awards

Dr J. E. Castle is the recipient of the Beilby Medal and Prize for 1977, the joint award of the Royal Institute of Chemistry, the Society of Chemical Industry and the Metals Society, for his work on the chemistry and metallurgy of surfaces, particularly studies of metallic corrosion by photoelectron spectroscopy.

Person to Person

Exchange furnished house in Richmond, Virginia, for similar in Cambridge, England from January 1978 to January 1979. Car exchange also. Contact Judith Bond, Department of Biochemistry, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298, USA.

Exchange 2-bedroom apartment in Jerusalem for similar in Bethesda area, Maryland, for one year beginning September/October 1977. Contact D. Ben Ezra, National Institutes of Health, Bldg 10, Room 10D-09, Bethesda, Maryland 20014, USA.

There will be no charge for this service. Send items (not more than 60 words) to Marcus Dobbs at the London office. The section will include exchanges of accommodation, personal announcements and scientific queries. We reserve the right to decline material submitted. No commercial transactions.

Meetings

12-14 September, **Conference on Scanning and Transmission Electron Microscopy**, Glasgow, UK (Institute of Physics, 47 Belgrave Square, London SW1, UK).

11-17 September, **VIIth European Congress of Pathology**, London, UK (Conference Services Ltd, 43 Charles Street, London W1, UK).

13-15 September, **Particle Size Analysis Conference**, Salford, UK (D. Dollimore, Reader in Physical Chemistry, University of Salford, Salford, UK).

12-16 September, **3rd International**

Conference on Bioindicators and Landscape Deterioration, near Prague, Czechoslovakia (Ustav krajinné ekologie CSAV (Ing. J. Spálený CSc.) 252 43 Pruhoňice, Czechoslovakia).

26-27 September, **Conference on Dynamic Small European Companies in the Health-Care Industry, and Why they are Successful**, Brussels (Robert S. First, Inc., Avenue Marnix, 19A, 1050 Brussels, Belgium).

12-14 October, **Working Group on Rehabilitation and Secondary Prevention in Patients with Acute Myocardial Infarction**, Valencia (WHO, Regional Office for Europe, 8 Schefvigsvej, DK-2100 Copenhagen, Denmark).

11-13 August, **Annual Meeting of the American Society of Pharmacognosy**, Washington (L.R. Brady, Pharmacy, University of Washington, Seattle, Washington 98195).

1-2 September, **The 571st Meeting of The Biochemical Society**, Dublin (Meetings Officer, The Biochemical Society, 7 Warwick Court, London, UK).

4-8 September, **3rd International Conference on Microprobe Analysis in Biology and Medicine**, Munster, West Germany (P. Echlin, Department of Botany, University of Cambridge, Cambridge, UK).

5-9 September, **European Conference on Precise Electrical Measurement**, Brighton (Institute of Electrical Engineers, Savoy Place, London, UK).

6-9 September, **7th European Microwave Conference and Microwave 1977**, Copenhagen (MEPL, Temple House, 34-36 High Street, Sevenoaks, Kent, UK).

6-9 September, **Conference on Digital Processing of Signals in Communications**, Loughborough (Conference Secretariat, Institution of Electronic and Radio Engineers, 8-9 Bedford Square, London, UK).

6-8 September, **Conference on Scaling for Performance Prediction in Rotodynamic Machines**, London (The Institution of Mechanical Engineers, 1 Birdcage Walk, London, UK).

8 September, **Seminar on Corrosion and Fatigue in Offshore Installations**, London (The Institution of Mechanical Engineers, 1 Birdcage Walk, London, UK).

12-15 September, **7th European Conference on Solid State Devices Research**, Brighton (Meetings Officer, Institute of Physics, 47 Belgrave Square, London, UK).

8 September, **The Draughting and Preparation of an Iso Standard on Nuclear Quality Assurance**, London (The Institution of Mechanical Engineers, 1 Birdcage Walk, London, UK).

13 September, **Mini-Computers—Applications**, London (The Institution of Mechanical Engineers, 1 Birdcage Walk, London, UK).

13–15 September, **Conference on Heat and Fluid Flow in Water Reactor Safety**, London (The Institution of Mechanical Engineers, 1 Birdcage Walk, London, UK).

14–16 September, **3rd European Conference on Optical Fibre Communication**, Munich (W. Harth, Lehrstuhl f. Allg. Elektrotechnik der Technischen Universität München, Arcisstrasse 21, D-8000 Munich 2, Germany).

14–16 September, **3rd National Conference on Quantum Physics**, Southampton (Meetings Officer, The Institute of Physics, 47 Belgrave Square, London, UK).

15 September, **Pressure Vessel Codes—Change and Advance**, London (The Institution of Mechanical Engineers, 1 Birdcage Walk, London, UK).

15–16 September, **Conference of the Schizophrenia Association of Great Britain**, London (Hon. Secretary, Tyr Twr, Llanfair Hall, Caernarvon, Wales).

Reports and Publications

UK & Ireland—June

Residential Short-term Care for Mentally Handicapped People: Suggestions for Action. (Pamphlet No. 4.) Pp. v+9. (London: National Development Group for the Mentally Handicapped, Alexander Fleming House, SE1, 1977.) £2.

Laboratory Practice Reviews of Current Techniques and Instrumentation. Edited by Bevan M. Gilpin. Pp. 82. (London: United Trade Press, Ltd., 42/43 Gerrard Street, W1, 1977.) £5.

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